Patrícia Costinha Marques

Degree in Biochemistry

The role of PGN hydrolases in the ability of *Staphylococcus aureus* to evade the host innate immune system

Dissertation to obtain the degree of Master in Biochemistry for Health

Orientador: Sérgio R. Filipe, Professor Auxiliar, FCT/UNL

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Visions of warm sunlight

The songs we wrote on old days

With laughter spreading far and wide

We are children dreaming awake

-Raphael and the Thornes

Resumo

Staphylococcus aureus é um microorganismo importante que pode ser encontrado na pele e membranas mucosas do corpo humano. Apesar da habilidade de colonizar o hospedeiro humano, a invasão deste patogénico para os tecidos pode originar severas condições de saúde. À medida que antibióticos actuais perdem eficiência contra este patogénico, torna-se urgente o desenvolvimento de novas estratégias de tratamento.

Peptidoglicano está presente em bactérias gram-negativas e gram-positivas, como uma molécula fundamental para a estrutura celular. Trata-se também de uma molécula que denuncia a presença da bactéria quando reconhecida por recetores inatos ao hospedeiro como proteínas PGRP.

De modo a evitar deteção, *S.aureus* desenvolveu estratégias para evadir o reconhecimento de PGN na superfície. Foi demonstrado que Atl (Atilano et al.), uma autolisina principal de *S.aureus,* é capaz de aparar os fragmentos mais externos de peptidoglicano de modo a impedir a ligação de PGRPs.

Este projecto foca-se no estudo da proteína SAOUHSC_00671 da estirpe *S.aureus* NCTC 8325-4, semelhante à Sle1, uma hidrolase capaz de aparar os fragmentos de PGN na divisão do septo da bactéria. Neste projeto focou-se à purificação da proteína SAOUHSC_00671 e à construção de mutantes *knock-out* de *S.aureus*, que carecem da sequencia codificante para SAOUHSC_00671.

Termos-chave: *Staphylococcus aureus*, peptidoglicano, autolisinas, Sle1, SAOUHSC_00671

Abstract

Staphylococcus aureus is an important microorganism that can be found on the skin and mucous membranes of the human body. Despite its ability to colonize the human host, invasion of this pathogen into the host tissue can lead to severe health conditions. As current antibiotics become less effective in the combat to this pathogen, it becomes urgent to develop new strategies of treatment.

Peptidoglycan is present in gram-negative and gram-positive bacteria alike, as a fundamental molecule for cell structure. It is a telltale molecule that betrays the presence of the bacteria when recognized by host innate immune receptors such as the PGRP proteins.

In order to avoid detection, S. aureus uses different strategies to circumvent recognition of the PGN at its surface. It has been shown that Atl, a major autolysin of S. aureus, is able to trim the outermost fragments of peptidoglycan in order to prevent the binding of PGRPs.

This project focused in studying the protein SAOUHSC_00671 from S. aureus NCTC 8324-5 strain, which belongs to the same protein family as SleI, a PGN hydrolase capable of trimming PGN fragments at the staphylococcal division septum. This project aimed purify the SAOUHSC_00671 protein and construct knock-out S. aureus mutants, which lack the coding sequence for SAOUHSC_00671.

Keywords: *Staphylococcus aureus*, peptidoglycan, autolysins, Sle1, SAOUHSC_00671.

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Abbreviations

- APS – Ammonium persulfate
- BSI – Bloodstream Infections
- CFU – Colony Forming Units
- ddH_2O – Bi-distilled water
- DNA – Deoxyribonucleic Acid
- dNTPs – Deoxyribonucleotides
- EDTA – Ethylenediamine tetra-acetic acid
- Fw - Foward
- g – Grams
- IE – Infective Endocarditis
- L - Liters
- LA – Luria-Bertani Agar
- LB – Luria-Bertani Broth
- M – Molar
- MCS – Multi Cloning Site
- mg – Milligrams
- mL – Milliliters
- MRSA – Methicillin-resistant *Staphylococcus aureus*
- nm – Nanometers
- OD – Over day
- OD_{600} – Optical Density at 600nm wavelength
- ON – Overnight
- PBP – Penicillin binding proteins
- PCR – Polymerase Chain Reaction
- PGRP – Peptidoglycan receptor proteins
- RPM – Rotations per minute
- RT – Room Temperature
- Rv - Reverse
- SA – *Staphylococcus aureus*
- SDS – Sodium Dodecyl Sulfate
- SDS/PAGE LB – Protein Loading Buffer
- ST – Sterile
- TAE – Tris- Acetate - EDTA
- TE – Tris-EDTA
- TSA – Tryptic Soy Agar
- TSB – Tryptic Soy Broth

- w/v – weight per volume
- WT – Wild type
- X-gal – 5-bromo-4-3-indolyl-β-D-galactopyranoside
- μg – Micrograms
- μL – Microliters

1 Introduction

1.1 Staphylococcus aureus

Staphylococcus bacteria are gram-positive cocci that are commonly found in the form irregular grape-like clusters. These organisms are widespread in nature, more specifically in the skin and mucous membranes, such as the mouth, mammary glands and intestinal, genitourinary, and upper respiratory tracts of mammals and birds [(1)]. The relationship established between the bacteria and the host is generally symbiotic, however, infiltration onto the host tissue can lead to severe health conditions such as skin infections, Bloodstream Infections (BSI) [(2)] and infective endocarditis [(3)].

The major incidence of *Staphylococcus aureus* related infections is in medical facilities [(4)]. Yilmaz et al. estimated that 42.1% of a series of 255 diagnosed cases of *Staphylococcus aureus* Bacteremia (SAB), were originated from vascular catheters and other surgical site infections. Healthcare associated SAB was defined in 55.7%.

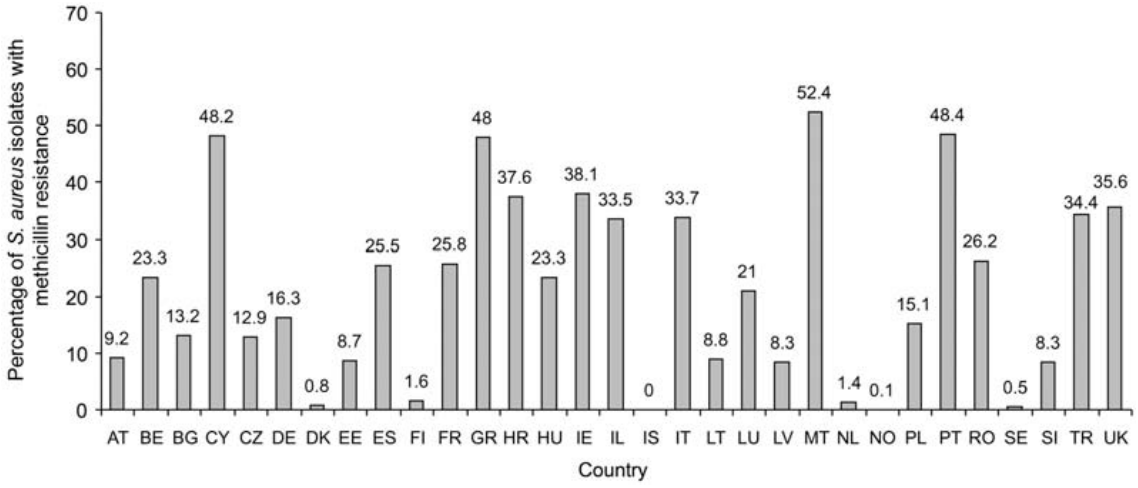

Figure 1.1 ***Prevalence of methicillin-resistant Staphylococcus aureus among patients with bacteremia in Europe in 2007, as reported by the European Antimicrobial Resistance Surveillance System*** [(5)]. AT, Austria; BE, Belgium; BG, Bulgaria; CY, Cyprus; CZ, Czech Republic; DE, Germany; DK, Denmark; EE, Estonia; ES, Spain; FI, Finland; FR, France; GR, Greece; HR, Croatia; HU, Hungary; IE, Ireland; IL, Israel; IS, Iceland; IT, Italy; LT, Lithuania; LU, Luxembourg; LV, Latvia; MT, Malta; NL, The Netherlands; NO, Norway; PL, Poland; PT, Portugal; RO, Romania; SE, Sweden; SI, Slovenia; TR, Turkey; UK, United Kingdom.

There is an increasing difficulty in treating these infections due to the acquired resistance of these organisms to antimicrobial drugs [(6,7)]. Klein et al. [(4)] estimated that the number of *S.aureus* infections resistant to methicillin increased 119% in north-American hospital facilities from 1999 to 2005. Reports from the European Antimicrobial Resistance Surveillance System stated an

increase of the annual rate of found Methicilin Resistant *S.aureus* (MRSA) isolates, in European countries during 1999-2006 [8].

S.aureus are majorly treated with administration of β-lactam antibiotics. These molecules bind to the native penicillin-binding proteins (PBPs) present in the cell wall interrupting the synthesis of peptidoglycan layer [9]. In methicillin-resistant strains, the gene *mecA* codifies for an additional, foreign, penicillin-binding protein (PBP2a) that prevents the binding of β-lactams and ensures the peptidoglycan synthesis is not disrupted [10]. Furthermore, MRSA tend to accumulate and develop resistances to other antibiotics [9,10].

1.2 Peptidoglycan and PGRPs

Peptidoglycan is the principal component of bacterial cell wall and is composed of long glycan chain made up of alternating amino sugars N-acetylglucosamine and N-acetylmuramic acid, and cross-linked by short peptides of variable length and composition [11].

In gram-positive bacteria, this peptidoglycan is covered by a series of different molecules, such as surface proteins, capsular polysaccharides [12] and wall-teichoic acids [13] (phosphate-rich glycopolymers involved in resistance to environments stress and regulation of bacterial division)

This peptidoglycan, however, can be recognized by peptidoglycan recognition proteins (PGRP), innate immunity molecules, as a pathogen-associated molecular pattern, or PAMP, and trigger inflammatory response against the bacterial pathogen [14].

Insects possess two response pathways able to discern between gram-positive and gram-negative bacteria (toll pathway for gram-positive bacteria, and Immune Deficiency pathway for gram-negative bacteria). When stimulation of these two pathways occurs, there are activated a series of responses, such as transcriptional regulators, antimicrobial peptides and signaling cascades, specific to each pathway [15].

In mammals, PGRPs can act as antibacterial agents due to their bactericidal and/or bacteriostatic activity, that is mediated by PGN hydrolytic activity [16], or by the binding of PGRPs to targets on the bacterial cell surface, which causes the activation of specific bacterial two-component systems [17].

How peptidoglycan is accessed by PGRPs is not yet fully known. PGN in gram-positive bacteria is concealed by layers of proteins and glycopolymers. Additionally, Atilano et al. [18] demonstrated that wall-teichoic acids play a vital role in limiting the access of Drosophila PGRP to bacterial peptidoglycan. It is therefore, inferred that bacteria may have developed strategies of evading the hosts PGRPs systems.

1.3 PGN Hydrolases

Bacteria organisms possess a variety of enzymes capable of cleaving bonds in polymeric peptidoglycan. These are known as hydrolases. They play vital role in regulating cell wall growth as well as other lysis phenomena. It has been documented the participation of PGN hydrolases in the release of turnover products from *E.coli* peptidoglycan during cell growth [19].

PGN hydrolases have different cleavage sites. N.Acetylmuramyl-L-alanine amidases disrupt the bond between MurNAc and N-terminal L-Alanine, thus separating the glycan strand from the peptide [20]. Carboxy- and endopeptidases hydrolyse amide bonds between amino acids. N-Acetyl-β-D-muramidades (or N-acetylmuramidases) cleave the bond β1,4-glycosidic between residues MurNAc and GlcNAc of peptidoglycan [20]. This can occur in two different ways: hydrolysis of the glycosidic bond by lysozymes results in terminal reducing MurNAc residue, whereas lytic transglycosylases enacts a transglycosylation reaction that forms a 1,6-anhydro ring at the MurNAc residue [20].

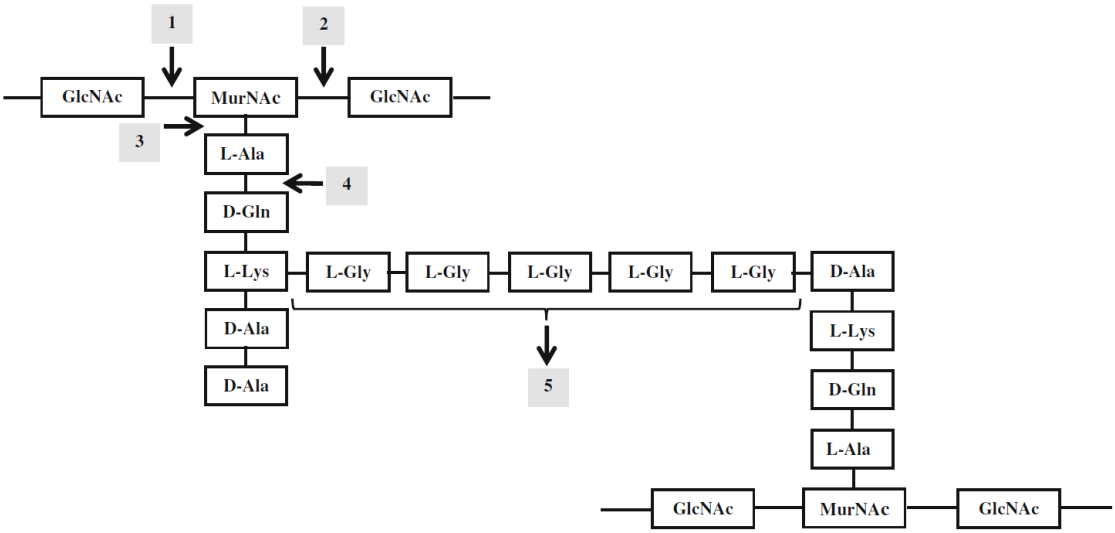

Figure 1.2: ***The structure of S. aureus peptidoglycan*** [11]. The arrows indicate the cleavage sites: 1 N-acetyl muramidase, 2 N-acetyl-β-D-glucosaminidase, 3 Nacetylmuramoyl-L-alanine amidase, 4 L-alanoyl-D-glutamate endopeptídases and 5 interpeptide bridge endopeptidases (e.g., lysostaphin). Abbreviations: GlcNAc N-acetylglucosamine, MurNAc N-acetylmuramic acid

The major autolysin of *S.aureus* is AtlA, composed of an amidase domain and a glucosaminidase domain that, upon proteolytic cleavage, yield two active proteins: amidase (AM) and glucosaminidase (GL) [21]. The role of this protein is essentially in cell division regulation but it has been documented the participation of AtlA in biofilm formation.

Additionally, Atilano et al. [22] has identified Atl as an essential protein in the concealment of bacterial PGN at the cell surface from host detection. In this study is shown that an autolysin-mediated "trimming" of PGN at cell surface, in which Atl partakes, renders the bacteria

inaccessible to host PGN receptors. As such, targeting bacterial autolysins to prevent their activity may constitute a new strategy against these pathogens.

The hydrolase Sle1 (also known as Aaa) has also been characterized [23,24]. Kajimura et al. [23] demonstrated this protein as a N-acetylmuramyl-L-alanine amidase and assessed its crucial role in the process of cell separation. Parallel to this study, Heilmann et al. [24] deduced the sequence of Sle1 from homologous DNA sequences from *S.aureus* strain Col, which revealed three identical domains highly homologous to the lysin motif (LysM) followed by a Chap domain in the C-terminal of the protein. Lysin motifs and Chap domains are often found in cell wall enzymes. [25,26,27]

1.3.1 *S.aureus* NCTC 8325-4 SAOUHSC_00671

Screening of the genome of *S.aureus* strain NCTC 8325-4 for similar proteins to Sle1, revealed two putative autolytic proteins: SAOUHSC_00671 and SAOUHSC_00773. The former revealed in its structure two LysM domains followed by a Chap domain, while the latter presented one LysM domain and a Chap domain.

The focus of this project will consist in the purification of the putative protein codified by SAOUHSC_00671 (abbreviated as gene 671 for this document) and the acquisition of knock-out mutants NCTC 8325-4 Δ671 via pMAD vector.

2 Materials and Equipment

All *S.aureus* and *E.coli* strains were provided from the research laboratory that hosted the student.

Cloning vector pET21a was chosen for the construction of the protein expression mutant for *E.coli* BL21(DE3). Gene 671 was cloned to the Multi Cloning Site (MCS) with primers NCTC671ClonFwXbaI (forward) and NCTC671ClonRvEagI (reverse), built *in-silico* with Lasergene's DNAStar software, SeqBuilder. Vector plasmid pET21a was provided from Novagen.

Shuttle vector pMAD (Arnaud et al., 2004) selected for the construction of the Δ671 mutants for *S.aureus* NCTC 8325-4. Regions 1000bp Up and 1000bp Down of gene 671 were cloned into the MCS.

All mutants and vectors *in-silico* sequences were built with Lasergene's DNAStar software, SeqBuilder, as well as the necessary primers. DNA template sequence of *S.aureus* NCTC 8325-4 was downloaded from National Center for Biotechnology Information (NCBI).

All restriction enzymes and their respective buffers were provided by New England Biolabs.

- 1kb plus DNA ladder (Thermo Scientific)
- 1kb GeneRuler DNA Ladder (Thermo Scientific, catalog number: SM0312
- 1 μl Loops (Sarstedt AG, catalog number: 86.1567.010)
- 25 ml Glass (or plastic disposable) Pipettes (Normax, catalog number: 4.5434334)
- 30% Acrylamide/Bis Solution (Bio-Rad Laboratories, catalog number: 161-0158)
- 50 ml Falcon tubes (Sarstedt AG, catalog number: 62.548.004)
- Ammonium persulfate (APS) [(NH4)2S2O8] (Sigma-Aldrich, catalog number: A3678)
- Bromophenol Blue sodium salt (Sigma-Aldrich, catalog number: B8026)
- Calcium chloride dihydrate (CaCl2.2H2O) (Sigma-Aldrich, catalog number: C3306)
- Clean Up Kit: Wizard SV Gel and PCR Clean-Up System (Promega Corporation, catalog number: A9285)
- DreamTaq Hot Start DNA Polymerase (Thermo Scientific, catalog number: EP1701)
- Ethanol (Merck Millipore Corporation, catalog number: 1.02371.1000)
- Glycerol (Sigma-Aldrich, catalog number: G8898)

- Hydrochloric acid (Merck Millipore Corporation, catalog number: 1.01834.2500)
- Luria Agar (Miller`s LB agar) (LA) (VWR, catalog number: 84684.0500)
- Luria Broth (Miller`s LB broth) (LB) (Sigma-Aldrich, catalog number: L3022)
- Lysostaphin (Sigma-Aldrich, catalog number: L7386)
- Magnesium chloride hexahydrate (Sigma-Aldrich, catalog number: M9272)
- Methylene Blue hydrate (Sigma-Aldrich, catalog number: 66720)
- Miniprep Kit: Wizard Plus SV Minipreps DNA Purification Systems (Promega Corporation, catalog number: A1465)
- Mini-PROTEAN® Comb, 10-well, 0.75 mm (Bio-Rad Laboratories, catalog number: 165-3354)
- N, N, N′, N′-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, catalog number: T9281)
- Nuclei Lysis Solution (Promega, catalog number: A7941)
- Petri dishes (Sarstedt AG, catalog number: 82.1473)
- Phusion High-Fidelity PCR Master Mix with HF buffer (Thermo Scientific, catalog number: F531S)
- Potassium hydroxide (KOH) (Sigma-Aldrich ,catalog number: P5958)
- Precision Plus ProteinTM Dual Color Standards (PPPS) (Bio-Rad Laboratories, catalog number: 1610374)
- Protein Precipitation Solution (Promega, catalog number: A7953)
- Rubidium Chloride, RbCl (Sigma-Aldrich, catalog number: 7791-11-9)
- Sodium chloride (Merck Millipore Corporation, catalog number: 1.06444.1000)
- Sodium Dodecyl Sulfate (Sigma-Aldrich, catalog number: L5750)
- Spacer Plates with 0.75 mm Integrated Spacers (Bio-Rad Laboratories, catalog number: 165-3310)
- Tris (Trizma® base) (Sigma-Aldrich, catalog number: T1503)
- Triton X-100 (Sigma-Aldrich, catalog number: T8787)
- Tryptic Soy Agar plates (TSA) (VWR, catalog number: 84602.0500)
- Tryptic Soy Broth (TSB) (BD, Bacto, catalog number: 211825)

3 Procedures and Protocols

3.1 *E.coli* competent cells

For the preparation of *E.coli* Dh5α, DC10b and BL21(DE3) competent cells was prepared the following solutions.

Table 3.1: ***Constitution of solutions RF1 and RF2****.*

RF1	**Total Volume 200mL**	**RF2**	**Total Volume 60mL**
RbCl	2,4 g	RbCl	0,072g
$MnCl_2$. 4 H_2O	1,98g	$CaCl_2$. 2 H_2O	0,66g
$CaCl_2$. 2 H_2O	0,30g	Glicerol	9g
Glycerol	30g	MOPS 0,5M pH 6,8	1,2 mL
KAc 1M pH 7,5	6 mL		

The pH of solutions RF1 and RF2 was corrected to 5,8 and 6,8, respectively and sterilized with a 0,22 μm filter. Solutions were stored at 4ºC until used.

E.coli strains Dh5α, DC10b and BL21(DE3) were inoculated in 5mL of LB medium and grown overnight at 37ºC with shaking.

The following day, 100μL of each overnight culture was retrieved and added to 100mL of LB medium. Cultures were incubated at 37ºC with shaking and grown until OD_{600}= 0,35 – 0,45. Upon reaching recommended OD, cultures were put on ice for 15 minutes. Samples were then transferred to falcon tubes of 50 mL and centrifuged at 4 ºC for 15 minutes at 3500 rpm.

Supernatant of centrifuged samples was discarded and each pellet was re-suspended in 15mL of RF1 solution. Falcons were placed on ice for 15 minutes and then centrifuged at 4ºC for 15 minutes at 3500 rpm. Supernatants were discarded and each pellet was re-suspended in 4mL of RF2 solution. Samples were placed on ice for 15 minutes.

800μL of glycerol 50% was added and the resulting solution was distributed into aliquots of 200μL each. Samples were stored at -80 ºC.

3.2 *S.aureus* genomic DNA extraction

S. aureus strain NCTC 8325-4 was plated in plates of TSA and grown overnight at 37 ºC.

Confluent growth was scrapped from overnight plates with the help of 10µL loops, and re-suspended in 50µL of EDTA pH 8. It was then added 1µL of Lysostaphin (10mg/mL) and 1µL of RNase (10mg/mL). Mixture was incubated at 37ºC for 30 minutes.

After incubation, it was added 200µL of 50mM EDTA followed by 250µL of Nuclei Lysis Solution. Mixture was incubated at 80ºC for 5 minutes and then cooled at room temperature.

It was then added 100µL of Protein Precipitation Solution to the solutions and vortex for 20 seconds. Samples were put on ice for 10 minutes and then centrifuged at top speed, for 20 minutes, at room temperature.

Resulting supernatant was transferred to a clean Eppendorf and added 420µL of Isopropanol. Samples were centrifuged again at top speed for 30 minutes.

Carefully, the supernatant was poured off and the tube cleaned with absorbent paper. Mixtures were added 250µL of 70% ethanol to wash the DNA pellet. It followed a new centrifugation at top speed for 3 minutes.

Ethanol was aspirated using a clean absorbent paper and air-dried for 5 minutes. DNA pellets re-suspended in 50µL of TE.

3.3 Amplification of gene 671

Gene 671 was amplified with the primers NCTC671ClonFwXbaI, GCGTCTAGACATCACCATCACCATCACTCTACACAACAT, and NCTC671ClonRvEagI, CGCGGCCGTTAATGGATGAATGCATAGC. Polymerase chain reaction was performed with the following Master Mix and reaction conditions:

Table 3.2: ***Content of Master Mix solution for 671 amplification****.*

Master Mix	**50µL**
HF Phusion Buffer 5x	10µL
dNTP (10mM)	1µL
Primer Fw (10mM)	1µL

Primer Rv (10mM)	1µL
Phusion enzyme	0,5µL
MilliQ H_2O	31,5µL
DNA template	1µL

Table 3.3: ***PCR conditions for 671 amplification.*** Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	98ºC	1:00
Denaturation	98ºC	0:10
Annealing	58ºC	0:30
Extension	72ºC	2:00
Final Extension	72 ºC	10:00
End	10 ºC	--

The resulting amplified product was loaded into a 0,8% agarose gel and separated at 120mV for 40 minutes. Sequentially, sample was cleaned with a Clean Up kit.

It followed the digestion of the purified insert, 35µL of DNA was digested with 1µL of restriction enzyme XbaI, 5µL of 10x NEBuffer CutSmart and water MiliQ to a total volume of 50µL. Mixture was kept overnight at a temperature of 37 ºC, after which, sample was cleaned with Clean Up kit and re-suspended on 50µL water MilliQ. From the cleaned mixture was retrieved a volume of 35µL and added 1µL of restriction enzyme EagI, 5µL of 10x NEBuffer 3.1 and water MilliQ to a total volume of 50µL. Digestion proceeded overnight at a temperature of 37 ºC, after which, mixture was purified with Clean Up kit and stored at a -20 ºC.

3.4 Construction of pET21a vector for protein expression

It was constructed two pET21a – 671 mutants intended for protein expression.

The first was denominated pET21a – 671 I and was constructed by digesting 35µL (5,103µg) of purified plasmid DNA with 1,5µL of restriction enzyme XbaI, 5µL of 10x NEBuffer CutSmart and water MilliQ to a total volume of 50µL. Digestion mixture was kept overnight at a

temperature of 37ºC, after which, sample was purified with Clean Up kit, and re-suspended in 50µL water MilliQ. From the purified sample was retrieved a volume of 35µL and added 1,5µL of restriction enzyme EagI, 5µL of 10x NEBuffer 3.1 and water MilliQ to a total volume of 50µL. Digestion was kept overnight at a temperature of 37 ºC, and purified with Clean Up kit afterwards. Re-suspended in 50µL of water MilliQ.

Ligase reaction was prepared with 1µL of T4 Ligase enzyme, 2µL of 10x Ligase buffer, 10µL of digested plasmid pET21a and 7µL of digested insert 671. Auto-ligated sample was prepared with the same volumes with the exception of digested insert 671, which was replaced with 7µL MilliQ water. Mixture was kept overnight at room temperature and stored at -20 ºC. In order to confirm a successful ligation, it was performed a PCR with the commercial primers *T7 promoter* and *T7 terminator*, and the enzyme DreamTaq. Master Mix solution were as follows:

Table 3.4: ***Content of Master Mix solution for pET21a-671 I verification.***

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer *T7 promoter* (20mM)	0,2µL
Primer *T7 terminator* (20mM)	0,2µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	14,1µL
DNA template	3µL

PCR conditions were as follows:

Table 3.5: ***PCR conditions for pET21a-671 I verification***. Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	3:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	1:30

Final Extension	72 ºC	5:00
End	10 ºC	--

Following the PCR reaction, the product was loaded on a 0,8% agarose gel with 5µL of RedSafe and separated at 120mV for 40 minutes.

The second plasmid was denominated pET21a – 671 II and was constructed by digesting 20µL (2,960µg) of purified plasmid DNA with 1,5µL of restriction enzyme NheI, 3µL of 10x NEBuffer 2.1 and water MilliQ to a total volume of 30 µL. Digestion mixture was kept overnight at a temperature of 37 ºC, after which, sample was purified with Clean Up kit, and re-suspended in 50µL water MilliQ. From the purified sample was retrieved a volume of 20µL (521,2 ng) and added 1µL of restriction enzyme EagI, 5µL of 10x NEBuffer 2.1 and water MilliQ to a total volume of 30µL. Digestion was kept overnight at a temperature of 37 ºC, and neutralized by heat inactivation at 65 ºC for 30 minutes.

Ligase reaction was prepared with 1µL of T4 Ligase enzyme, 2µL of 10x Ligase buffer, 3µL (3,909µg) of digested plasmid pET21a and the following volumes of digested insert 671: 10µL, 7µL, 5µL and 0µL (auto-ligated plasmid). Mixture was kept overnight at room temperature and stored at -20 ºC.

In order to confirm a successful ligation, it was performed a PCR with the commercial primers T7 promoter and T7 terminator, and the enzyme DreamTaq. Master Mix solution were as follows:

Table 3.6: ***Content of Master Mix solution for pET21a-671 II verification***.

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer *T7 promoter* (20mM)	0,2µL
Primer *T7 terminator* (20mM)	0,2µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	14,1µL

DNA template	3µL

PCR conditions were as follows:

*Table 3.7**: PCR conditions for pET21a-671 II verification.*** Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	5:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	2:30
Final Extension	72 ºC	5:00
End	10 ºC	--

PCR product was loaded on 0,8% agarose gel with 5µL RedSafe and separated at 120mV for 40 minutes.

3.5 *E.coli* transformation

Competent cells of the strain Dh5α were prepared according to the protocol previously described (Section 3.1). Cells were retrieved from –80ºC storage and thawed in ice. Once defrosted it was added 5µL of ligated DNA to the cell aliquots and placed on ice for 10 minutes.

Sequentially, samples were incubated at 42 ºC for 1 minute and then immediately placed on ice for 5 minutes. After ice incubation, it was added 1mL LB to cell samples and incubated under agitation at 37 ºC for 1 hour.

Samples were plated in LA plates supplemented with 100µg/mL of Ampicillin and incubated overnight at 37 ºC.

3.6 Colony Screening PCR

Transformed colonies of *E.coli* were confirmed via PCR. From ON plates was streaked several isolated colonies into fresh LA plates supplemented with 100µg/mL of Ampicillin and incubated overnight. The grown colonies were streaked with the resource of a wooden toothpick directly into the PCR tube.

PCR reaction was performed with the commercial primers T7 promoter and T7 terminator, and the enzyme DreamTaq. Master Mix solution were as follows:

Table 3.8: ***Contents of Master Mix solution for Colony Screening PCR of E.coli colonies***.

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer T7 promoter (20mM)	0,2µL
Primer T7 terminator (20mM)	0,2µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	17,1µL

PCR conditions were as follows:

Table 3.9: ***PCR conditions for Colony Screening PCR for E.coli colonies.*** Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	10:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	2:30
Final Extension	72 ºC	5:00
End	10 ºC	--

PCR product was run on 0,8% agarose gel with 5µL of RedSafe and separated at 120mV for 40 minutes.

3.7 *E.coli* BL21(DE3) induction test

E.coli strain BL21(DE3) was transformed with pET21a plasmid according to stated transformation protocol (Section 3.5). One isolated colony was inoculated in 3ml of LB medium supplemented with 100µg/ml Ampicillin and incubated overnight at 37ºC under agitation.

Grown ON culture was diluted to 0,05 OD_{600} in 20mL of LB medium, supplemented with 100µg/mL, incubated at a temperature of 37 ºC and agitation of 150 rpm until it reached 0,5 OD_{600}.

When culture reached recommended OD_{600}, it was divided in equal volume to another Erlenmeyer and added 1mM of IPTG. Nothing was added to the initial Erlenmeyer (negative control).

Both cultures proceeded with incubation at 37 ºC under agitation of 150 rpm. Aliquots of each cell culture were retrieved and stored at -20 ºC.

3.8 SDS-PAGE Gel Electrophoresis

Acrylamide gel matrix for SDS-PAGE gel electrophoresis was prepared following:

Table 3.10: ***Preparation of the Resolution Gel with 12,5% of Acrylamide****.*

Resolution Gel 12,5% Acrylamide	**Total Volume 10mL**
MilliQ H_2O	3,4mL
Tris-HCl 1,5M pH 8,8	2,48ml
Acrylamide	4,1mL
SDS 10% (w/v)	100µL
APS 10% (w/v)	50µL
TEMED	5µL

Table 3.11: ***Preparation of the Stacking Gel with 4% Acrylamide.***

Stacking Gel 4% Acrylamide	**Total Volume 3mL**
MilliQ H_2O	1,8mL
Tris-HCl 0,5M pH 8,8	750µL
Acrylamide	400µL
SDS 10% (w/v)	30µL
APS 10% (w/v)	15µL
TEMED	3µL

Samples were centrifuged for 5 minutes at top speed, re-suspended in Protein Loading Buffer 1x and boiled for 5 minutes before loading in gel. Protein Loading Buffer solution was prepared as followed:

Table 3.12: ***Preparation of SDS/Page Protein Loading Buffer 5x****. Sample stored at -20ºC.*

Protein Loading Buffer 5x	**Total Volume 5mL**
SDS 10% (w/v)	1mL
Glicerol	0,5mL
Tris 1M pH 6,8	0,8mL
Bromophenol Blue 0,2% (w/v)	0,1mL
β-Mercaptoethanol	0,5mL
MilliQ H_2O	2,1mL

Band separation was performed at 80mV in 1x Tris-Glycine SDS Running Buffer for approximately two hours. Gel was stained in Blue Safe overnight.

3.9 Sanger Sequencing

Sanger sequencing was provided by STABvida Laboratories, in FCT UNL campus. (http://www.stabvida.com)

3.10 *S.aureus* competent cells

For the preparation of *S.aureus* competent cells was prepared a solution of Sucrose 0,5M, filtered and stored at 4ºC until used.

S.aureus strain RN4220 was inoculated in 5mL of TSB medium and grown overnight at 37ºC.

The following day, overnight culture was diluted 1:200 ratio into 100mL of TSB medium and incubated at 37ºC with shaking until OD_{600}= 0,4-0,6. Upon reaching recommended OD, cultures were transferred into four pre-cooled 50mL Falcon tubes and centrifuged at top speed, 4ºC for 15 minutes.

Supernatant of centrifuged samples was discarded and pellet re-suspended in 50mL of sucrose 0,5M. Samples were centrifuged again at top speed, 4ºC for 15 minutes. Supernatant was discarded and pellet re-suspended 25mL of sucrose 0,5M.

The content of each two Falcon tubes were combined, placed on ice for 15 minutes and centrifuged again for 15 minutes at 4ºC.

Pellets were re-suspended in 300µL of sucrose 0,5M and all the content was combined into one single Falcon tube. The resulting mixture was distributed into aliquots of 50µL and stored at -80ºC.

3.11 Purification of Up and Down regions of gene 671

Regions 1kb Upstream and 1kb Downstream of gene 671 were amplified from genomic DNA extracted from *S.aureus* NCTC 8325-4 with the following primers:

Table 3.11: ***Forward and reverse primers for amplification of fragments Up and Down***

Up	
NCTC671UpFwEcoRI (P1)	CGC GCG AAT TCT GGG TAT TAT TAC GTT GGC ATT G
NCTC671UpRegOver (P2)	GGA GTG CCT TTG TAA TTA AAT AAA TTG TAC TG
Down	
NCTC671DwRegOver (P3)	CAA TTT ATT TAA TTA CAA AGG GAC TCC TCC
NCTC671DwRevBamHI (P4)	CGC GGA TCC TTC TGA ACA TAT TAG CAA GGT AAA G

Master Mix constitution and reaction conditions for both PCRs are as follows:

Table 3.12: ***Contents of Master Mix solution for Up and Down fragments amplification.***

Master Mix	**50µL**
HF Phusion Buffer 5x	10µL
dNTP (10mM)	1µL
Primer Fw (P1 or P3) (10mM)	1µL
Primer Rv (P2 or P4) (10mM)	1µL
Phusion enzyme	051µL
MilliQ H_2O	31,5µL

DNA template	1µL

Table 3.13: ***PCR conditions for Up and Down cloning PCR***. Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	98ºC	1:00
Denaturation	98ºC	0:10
Annealing	58ºC	0:30
Extension	72ºC	2:00
Final Extension	72 ºC	10:00
End	10 ºC	--

After PCR, all samples were loaded into a 0,8% agarose gel with 5µL RedSafe and separated at 120mV for 40 minutes. Afterwards, the amplified product was cleaned using a Clean Up kit.

Once inserts Up and Down were purified, it followed an Overlapping PCR with primers NCTC671UpFwEcoRI (P1) as forward primer and NCTC671DwRevBamHI (P4) as reverse primer. The resulting amplification will consist of inserts Up and Down combined into a single, longer DNA fragment (UP-DOWN). PCR conditions and Master Mix constitution were as follows:

Table 3.14: ***Contents of Master Mix solution for overlapping PCR of Up and Down fragments.***

Master Mix	**50µL**
HF Phusion Buffer 5x	10µL
dNTP (10mM)	1µL
Primer Fw P1 (10mM)	1µL
Primer Rv P4 (10mM)	1µL
Phusion enzyme	0,5µL
MilliQ H_2O	30,5µL

DNA template Up	1µL
DNA template Down	1µL

*Table 3.15**: PCR conditions for Overlapping PCR of Up and Down regions.*** Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	98ºC	1:00
Denaturation	98ºC	0:10
Annealing	58ºC	0:30
Extension	72ºC	4:00
Final Extension	72 ºC	10:00
End	10 ºC	--

After PCR, all samples were loaded into a 0,8% agarose gel with 5µL RedSafe and separated at 120mV for 40 minutes. Amplified band was cut from the gel and cleaned with a Clean Up kit.

It followed the sequential digestion of the purified UP-DOWN insert. For the first reaction 6,35µL (800ng) of insert was digested with 1µL of restriction enzyme EcoRI, 3µL of 10x NEBuffer 3.1 and water MiliQ to a total volume of 30µL. Mixture was kept overnight at a temperature of 37 ºC, after which, sample was cleaned with Clean Up kit and re-suspended on 50µL water MilliQ, From the cleaned mixture was retrieved a volume of 20µL and added 1µL of restriction enzyme BamHI, 5µL of 10x NEBuffer 3.1 and water MilliQ to a total volume of 50µL. Digestion proceeded overnight at a temperature of 37 ºC, after which, mixture was purified with Clean Up kit and stored at a -20 ºC.

3.12 Construction of pMAD vector for gene excision

The plasmid was denominated pMAD del 671 and was constructed by digesting 20µL (1,066µg) of purified plasmid DNA with 1µL of restriction enzyme EcoRI, 5µL of 10x NEBuffer 3.1 and water MilliQ to a total volume of 30µL. Digestion mixture was kept overnight at a temperature of 37 ºC, after which, sample was purified with Clean Up kit, and re-suspended in 50µL water MilliQ. From the purified sample was retrieved a volume of 20µL and added 1µL of restriction enzyme BamHI, 5µL of 10x NEBuffer 3.1 and water MilliQ to a total volume of 50µL.

Digestion was kept overnight at a temperature of 37 ºC, and cleaned with Clean Up kit afterwards. Re-suspended in 50µL of water MilliQ.

Ligase reaction was prepared with 1µL of T4 Ligase enzyme, 2µL of 10x Ligase buffer, 10µL (67ng) of digested plasmid pMAD and the following volumes of digested insert UP-DOWN: 10µL, 7µL, 5µL and 0µL (auto-ligated plasmid). Mixture was kept overnight at room temperature and stored at -20 ºC.

To confirm a successful ligation, it was performed a PCR with the primers pMAD1 (forward) and pMAD2 (reverse). PCR conditions and Master Mix constitution are as follows:

Table 3.16: ***Contents of Master Mix solution for pMAD del 671 verification***.

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer pMAD1 (10mM)	0,4µL
Primer pMAD2 (10mM)	0,4µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	16,7µL
DNA template	3µL

Table 3.17: PCR conditions for pMAD del 671 verification. Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	3:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	3:00
Final Extension	72 ºC	5:00
End	10 ºC	--

PCR product was loaded on a 0,8% agarose gel with 5µL of RedSafe and separated at 120mV for 40 minutes.

Once verified a successful ligation of pMAD del 671, the plasmid was transformed to *E.coli* strain of DC10b according to the *E.coli* transformation protocol (section 3.5). Transformed colonies were screened via Colony Screening PCR (section 3.6) with the primers pMAD1 and pMAD2. Plasmid DNA was extracted with a Miniprep kit from positive colonies and stored at -20ºC.

3.13 *S.aureus* electroporation

S.aureus competent cells were thawed in ice and 5µL of purified DNA plasmid was added to the aliquot. Mixture was then transferred to a 0,2 cm BioRad Gene Pulser cuvette and incubated on ice for 5 minutes.

Samples were then retrieved and pulsated once at 2500V, 25µF, 100Ω for 2,5 msec. Cells were immediately rescued with 1mL of TSB medium and placed on ice for 15 minutes. Samples were then incubated at 30 ºC, under agitation for 2 hours.

3.14 Transduction protocol

The following solutions were prepared:

Table 3.20: ***Preparation of 0,3GL Top and Bottom Agar***. Medium was autoclaved at 120ºC for 40 minutes.

0,3GL Top Agar (Bottom Agar) pH 7,8	**Concentration**
Casamino Acids	3 g/L
Yeast Extract	3 g/L
NaCl	5,9 g/L
Sodium Lactate, 60% syrup/DL-Lactate Acid	3,3 mL/L
50% Glicerol	2 mL/L
Tri-Sodium Citrate	0,5 g/L
Agar for Top agar (for Bottom agar)	7,5 g/L (15 g/L)

Table 3.21: ***Preparation of Phage Top and Bottom Agar***. Medium was autoclaved at 120ºC for 20 minutes.

Phage Top Agar (Bottom Agar) pH 7,8	**Concentration**
Casamino Acids	3 g/L

Yeast Extract	3 g/L
NaCl	5,9 g/L
Agar for Top agar (for Bottom agar)	5 g/L (15 g/L)

Table 3.8: ***Preparation of Phage Buffer.*** Solution as sterilized with 0,22 μm filter.

Phage Buffer	**Concentration**
0,1M $MgSO_4$	10 mL/L (1mM)
0,4M $CaCl_2$	10 mL/L (4mM)
2,5M Tris pH 7,8	20 mL/L (50mM)
NaCl	5,9 g/L
Gelatin	1 g/L

All media and buffers were stored at 4ºC.

In order to make the phage lysate, a single colony of transformed *S.aureus* strain RN4220 was plated and incubated at 30ºC overnight.

It was retrieved two 10μL loops of confluent growth and re-suspended in 1mL of TSB medium where it was added 5mM of $CaCl_2$.

Several dilutions of Phage 80α lysate were prepared with Phage Buffer and kept at 4ºC. Additionally, it was prepared 3mL of Phage Top Agar supplemented with 5mM $CaCl_2$, 10μL of cell suspension and 10μL of diluted phage lysate. The mixture was poured onto 20mL Bottom Phage Agar plates sustained with 5mM of $CaCl_2$. Plates were incubated overnight at 30ºC.

The following day, 4mL of Phage Buffer was added to plates showing confluent lysis and place at 4ºC for one hour. Sequentially, the Phage Top Agar layer was collected into a Falcon tube, vortexed and incubated at 4ºC for one hour. Mixture was then centrifuged at top speed for 15 minutes at 4ºC.

Supernatant was recovered and filtered with a 0,45μm filter and stored at 4ºC. As a sterility control, 50μL of recovered phage lysate was plated on a TSA plate and incubated overnight at 37ºC.

For the transduction procedure it was plated a single colony of the receiving strain of *S.aureus* NCTC 8325-4 and incubated overnight at 30ºC.

The following day it was re-suspended two loops of the confluent growth in 1mL of TSB medium supplemented with 5mM $CaCl_2$.

It was poured into petri dishes 10mL of 0,3GL Bottom Agar containing 3x 10µg/mL Erythromycin. After agar is set, it was poured a new layer of 20mL 0,3GL Bottom Agar without antibiotic.

The following transduction mixtures were prepared:

Table 3.23: ***Transduction mixtures prepared****.*

Sample	**Culture (µL)**	**Phage Lysate (µL)**	**Phage Buffer µL**
1	100	0,1	100
2	100	1	100
3	100	10	100
4	100	100	100
Control	100	-	100

Mixtures were incubated under agitation for 20 minutes at 37ºC.

Each transduction mixture was added to 3mL of Top Agar in a test tube, shaken and immediately poured onto a prepared 0,3GL Bottom Agar plate. After solidified, plates were incubated at 30ºC for 48 hours.

3.15 Gene knock-out protocol

For the integration step of gene knock-out procedure, inoculates of transduced strain of *S.aureus* NCTC 8325-4 + pMAD del 671 were prepared in TSB medium supplemented with 10µg/ml of Erythromycin and incubated overnight at 30ºC. The following day, cultures were diluted 1:1000 in fresh medium of TSB with 10µg/mL Erythromycin and incubated at 30ºC for a period of 8 hours. After incubation over day, cultures were once again diluted at 1:1000 ratio in TSB medium with 10µg/mL Erythromycin and incubated at 43ºC overnight.

The following day, grown cultures were plated in fresh plates of TSA medium supplemented with 10µg/mL Erythromycin and 100µg/mL X-Gal with the following dilutions: 10^{-4}, 10^{-5} and 10^{-6}. Plates were then incubated overnight at 43ºC. The following day, isolated blue colonies were stricken to fresh plates of TSA + 10µg/mL Erythromycin + 100µg/mL X-Gal and incubated at 43ºC overnight.

Successful integration was confirmed via PCR of the genomic DNA extracted from the stricken blue colonies (PCR conditions and procedure of genomic DNA extraction listed below, sections 3.17 and 3.16, respectively).

Positive colonies with integrated pMAD del 671 plasmid were selected for the excision step of gene knock-out procedure. Samples were inoculated in TSB medium with no antibiotic and incubated at 30ºC overnight. The following day, cultures were diluted 1:500 in fresh TSB medium with no antibiotic and incubated over day at 30ºC for a period of 8 hours. Afterwards, cultures were diluted 10^{-4}, 10^{-5} and 10^{-6} and plated in fresh plates of TSA + 100µg/mL X-Gal. Plates were incubated at 43ºC overnight.

The following day, the same isolated white colonies were stricken in fresh plates TSA + 10µg/mL Erythromycin + 100µg/mL X-Gal as well as plates TSA + 100µg/mL X-Gal. Both set of plates were incubated overnight at 37ºC.

After incubation, white colonies that did not grow in TSA medium supplemented with Erythromycin were stricken into fresh plates of TSA + 100µg/mL X-Gal and incubated overnight at 37ºC. The following day, genomic DNA of grown white colonies was extracted (section 3.16) and a PCR (section 3.17) was performed to confirm the successful excision of the gene 671.

3.16 *S.aureus* genomic DNA extraction for PCR screening.

The following solutions were prepared:

- Alkaline Wash Solution
    - 0,05M Sodium Citrate
    - 0,5M NaOH
- 0,5M Tris-HCl pH 8,0

Cells were scraped from plates and re-suspended in 500µL of Alkaline Wash Solution and followed by room temperature incubation for 20 minutes. Sequentially, samples were centrifuged at top speed for 1 minute and the resulting pellet was washed with 500µL of 0,5M Tris-HCl pH 8,0. A new centrifugation was performed and pelleted cells were re-suspended in 100µL of MilliQ water.

Samples were boiled for 10 minutes followed by centrifugation at top speed for 5 minutes. Supernatant was then transferred to a new, clean Eppendorf and stored at -20ºC.

3.17 PCR screening for gene knock-out confirmation

A successful integration of the construct pMAD del 671 was verified by performing two separated PCR screenings with two different sets of primers, of the extracted genomic DNA samples. The first PCR screening was performed with the set of primers forward

"NCTC671UpFwEcoRI" (P1) and reverse pMAD2. The resulting amplification will verify if the crossing over took place on the Up region of the 671 gene. The second set of primers consisted of the forward primer pMAD1 and the reverse primer "NCTC671DwRevBamHI" (P4). This set of primers will amplify for successful crossing over on the Down region of the gene 671.

The Master Mix constitution and PCR conditions for integration screening are as follows:

Table 3.24: ***Contents of Master Mix solution for integration screening PCR***.

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer P1/pMAD1 (10mM)	0,4µL
Primer pMAD2/P4 (10mM)	0,4µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	14,1µL
Genomic DNA	3µL

Table 3.25: ***PCR conditions for integration screening PCR***. Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	10:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	3:00
Final Extension	72 ºC	10:00
End	10 ºC	--

After PCR, all samples were loaded into a 0,8% agarose gel with 5µL RedSafe and separated at 120mV for 40 minutes.

For the verification of a successful excision of the gene 671 was performed a PCR screening with the primers "NCTC671UpFwEcoRI" (P1) as forward primer and

"NCTC671DwRevBamHI" (P4) as reverse primer. The constitution of the reaction Master Mix and the PCR conditions are as follows:

Table 3.26: ***Contents of Master Mix solution excision verification PCR.***

Master Mix	**20µL**
DreamTaq Green Buffer 10x	2µL
dNTP (10mM)	0,4µL
Primer P1 (10mM)	0,4µL
Primer P4 (10mM)	0,4µL
DreamTaq enzyme	0,1µL
MilliQ H_2O	14,1µL
Genomic DNA	3µL

*Table 3.27****: PCR conditions for excision verification PCR***. Number of cycles (a cycle comprising the steps of Denaturation, Annealing and Extension) was of 30.

Step	**Temperature**	**Time (min)**
Initial Denaturation	95 ºC	10:00
Denaturation	95 ºC	0:30
Annealing	53 ºC	0:30
Extension	72 ºC	2:00
Final Extension	72 ºC	10:00
End	10 ºC	--

After PCR, all samples were loaded into a 0,8% agarose gel with 5µL RedSafe and separated at 120mV for 40 minutes.

4 Results and Discussion

4.1 Construction of pET21a-671 I

As it was described in section 3.3, gene 671 was amplified from the extracted DNA of *S.aureus* strain NCTC 8325-4. PCR reaction amplified a DNA fragment of approximately 800bp which is in conformance with the *in-silico* sequence. As figure 4.1 demonstrates, the amplified band is inferior to the 850bp ladder band and shows no other amplification products. Control sample was performed with MilliQ water (last lane before ladder) and it displays no amplification.

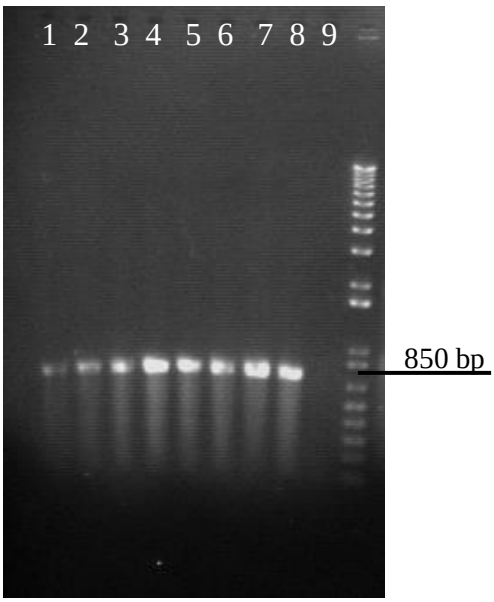

Figure 4.1: ***Amplification of gene 671.*** Expected length of amplified fragment is approximately 850bp. 1 to 8- Amplified 671 insert. 9- control.

Cloning vector pET21a was extracted using a Miniprep kit from *E.coli* strain Dh5α and digested with restriction enzymes XbaI, EagI and NheI.

In order to assess the integrity of the extracted and digested DNA, it was performed an agarose gel (Figure 4.2) where pET21a non-digested was loaded next to the digested samples. All lanes display two bands with no visible smears: the first band at 5000bp is in accordance to the expected size of pET21a (which has approximately 5500bp) whereas the second band over the 12000bp ladder mark corresponds to super-coiled form of DNA.

All used enzymes have a unique restriction site that will linearize the circular plasmid, and therefore display a band of the same weight as the circular plasmid. The existence of the second band from the super-coiled form confirms an incomplete digestion.

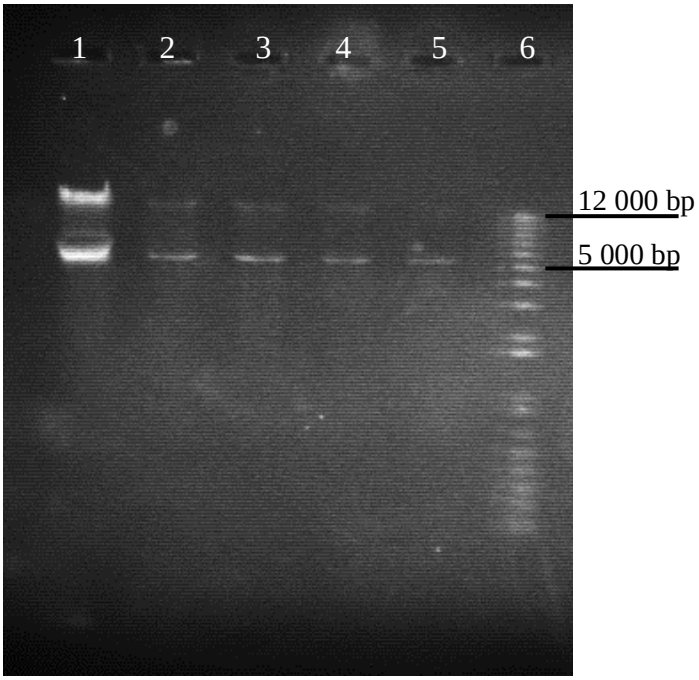

Figure 4.2: ***Plasmid pET21a digested with restriction enzymes XbaI, EagI and NheI***. 1- Plasmid pET21a. 2- pET21a digested with XbaI. 3- pET21a digested with EagI. 4- pET21a digested with NheI. 5- pET21a digested with XbaI and EagI.

Digested plasmid and insert 671 were ligated using a T4 Ligase enzyme. Considering the gene 671 was inserted in the multiple cloning site of pET21a it is possible to verify a successful ligation with the primers *T7 promoter* and *T7 terminator*, located upstream and downstream of the MCS. PCR conditions are described in section 3.4 and the agarose gel is shown in figure 4.3.

The amplification product of a successful ligation will consist of a DNA fragment with the approximate size of 850bp, as is shown in lane 1 of figure 4.3. The auto-ligated plasmid (lane 2) will amplify a DNA fragment of 300bp.

A plasmid digested with either XbaI, EagI or both enzymes will become linear and will not allow any amplification. The existence of a 300bp band in lane 3 indicates the digestion of pET21a with either XbaI or EagI was incomplete and the plasmid returned to its original circular form.

In lane 6 is displayed the amplification of 300bp fragment that is expected of pET21a plasmid.

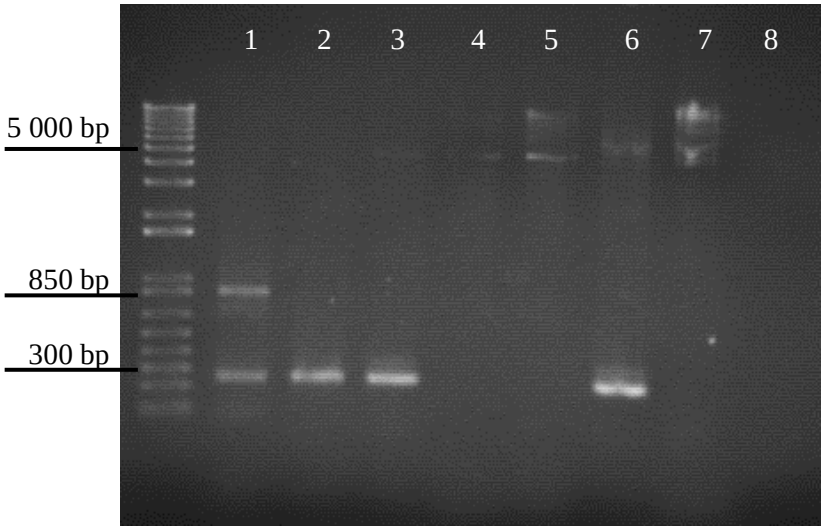

Figure 4.3: ***Cloning PCR of pET21a-671 I plasmid****.* Expected length of the amplified 671 insert is of 850bp, which verifies a successful insertion. 1- PCR product of pET21a-671 I. 2-PCR product of auto-ligated pET21a. 3- PCR product of pET21a digested with XbaI and EagI. 4- pET21a digested with XbaI and EagI. 5- pET21a digested with Xbal. 6- PCR product of pET21a. 7- pET21a. 8- Control.

4.2 Positive colonies of pET21a-671 I.

The confirmed ligated pET21a-671 I was transformed into *E.coli* strain Dh5α according to the protocol described in section 3.5. The auto-ligated pET21a was also transformed. Several of the resulting colonies were stricken into fresh plates and screened via Colony Screening PCR (section 3.6)

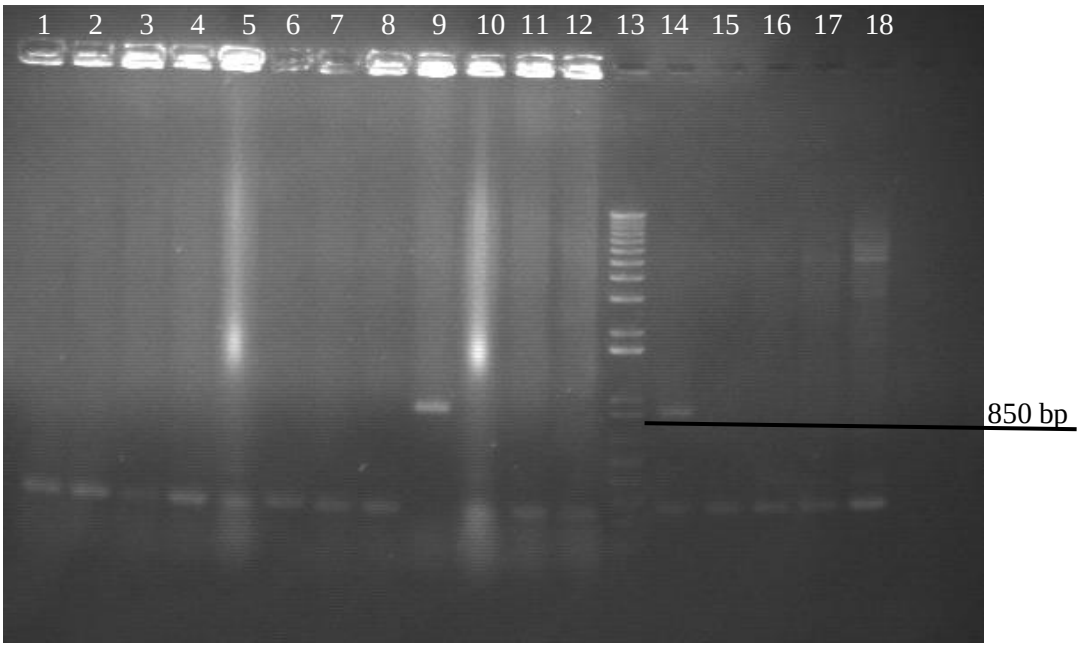

Figure 4.4: ***Screening PCR for pET21a-671 I positives****.* 1 to 11- Amplification from transformed Dh5α with pET21a-671 I. 12- Amplification from transformed Dh5α with auto-ligated pET21a. 13- DNA ladder 14- Amplification from pET21a-671 I (positive control). 15- Amplification from auto-ligated pET21a (negative control). 16- Amplification from pET21a digested with XbaI and EagI. 17- Amplification from pET21a digested with XbaI. 18- Amplification from pET21a.

PCR conditions and Master Mix constitution were as it is described in section 3.6. From a selection of 11 transformed Dh5α colonies with plasmid pET21a-671 I, only one showed the amplification of the 800bp band respective to gene 671 (figure 4.4).

Positive colony of pET21a-671 I was stricken into fresh plate and plasmid DNA was extracted with a Miniprep kit. It followed a digestion with restriction enzyme NaeI in order to assess a Digestive Profile. Enzyme NaeI possesses three restriction sites as it is demonstrated in figure 4.5.

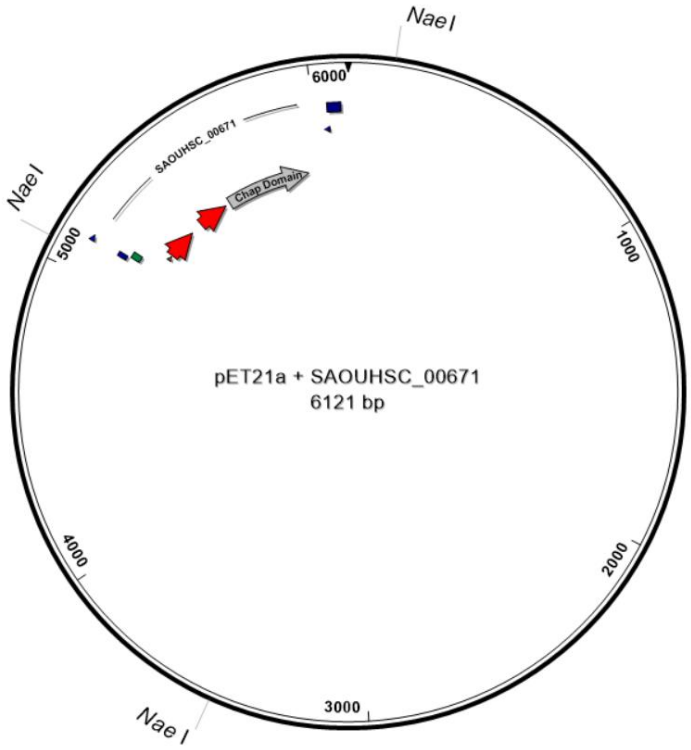

Figure 4.5: ***Restriction sites of NaeI in plasmid pET21a-671 I.***

It was digested 1µg of plasmid pET21a-671 I and plasmid pET21a that served as negative control.

As it is shown in table 4.1, both plasmids will be digested into three DNA fragments however, the smallest fragment of the empty pET21a (456bp) will have a significant size difference when compared to the same fragment of pET21a-671 I (1197bp). This difference is due to the inserted 671 gene and will be visible when separated by gel electrophoresis.

Table 4.1: ***Size comparison of DNA fragments produced by NaeI digestion****.*

pET21a	**pET21a-671 I**
3336	3336
1588	1588
456	***1197***

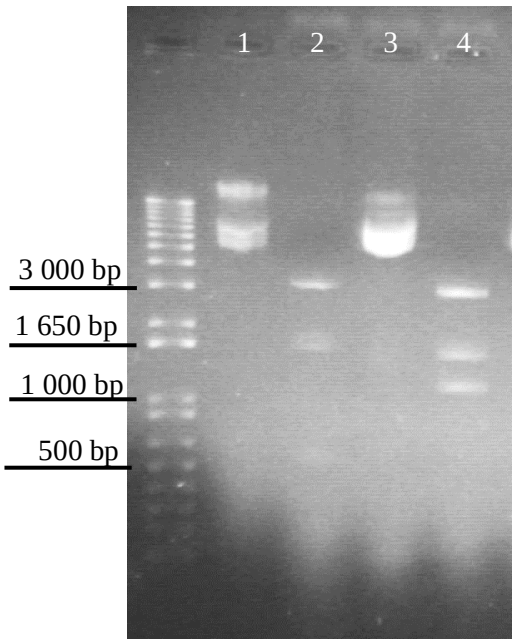

Figure 4.6: ***Digestive profile of pET21a-671 I by NaeI.*** Digestion with NaeI enzyme will result in three fragments for both pET21a-671 I and pET21a. The size difference in the smallest fragment attests for a positive insertion of gene 671. 1- pET21a non-digested. 2- Digested pET21a. 3- pET21a-671 I non-digested. 4- Digested pET21a-671 I.

The agarose gel in figure 4.6 depicts the digestion of both plasmids by NaeI. As it was expected, the two plasmids display two bands of equal size: a first with 3000bp followed by the second close to the 1650bp ladder band.

The last band of the sample pET21a is barely visible in the agarose gel but is clearly below the 1000bp ladder mark. The positive sample pET21a-671 I, however, exhibits the third band above the 1000bp which confirms the insertion of gene 671.

4.3 Induction test of pET21a-671 I

Positive plasmid pET21a-671 was transformed into *E.coli* strain BL21(DE3) as it was described in section 3.5. One transformed colony was selected and inoculated in LB medium supplemented with 100µg/ml Ampycilin, incubated overnight at 37ºC, 150rpm.

Protein induction test was performed according to section 3.7, with 1mM IPTG and temperature 37ºC. Aliquots were retrieved at time 0 of induction, at 3-hour induction and overnight induction. Optical density was also measured at the mentioned points.

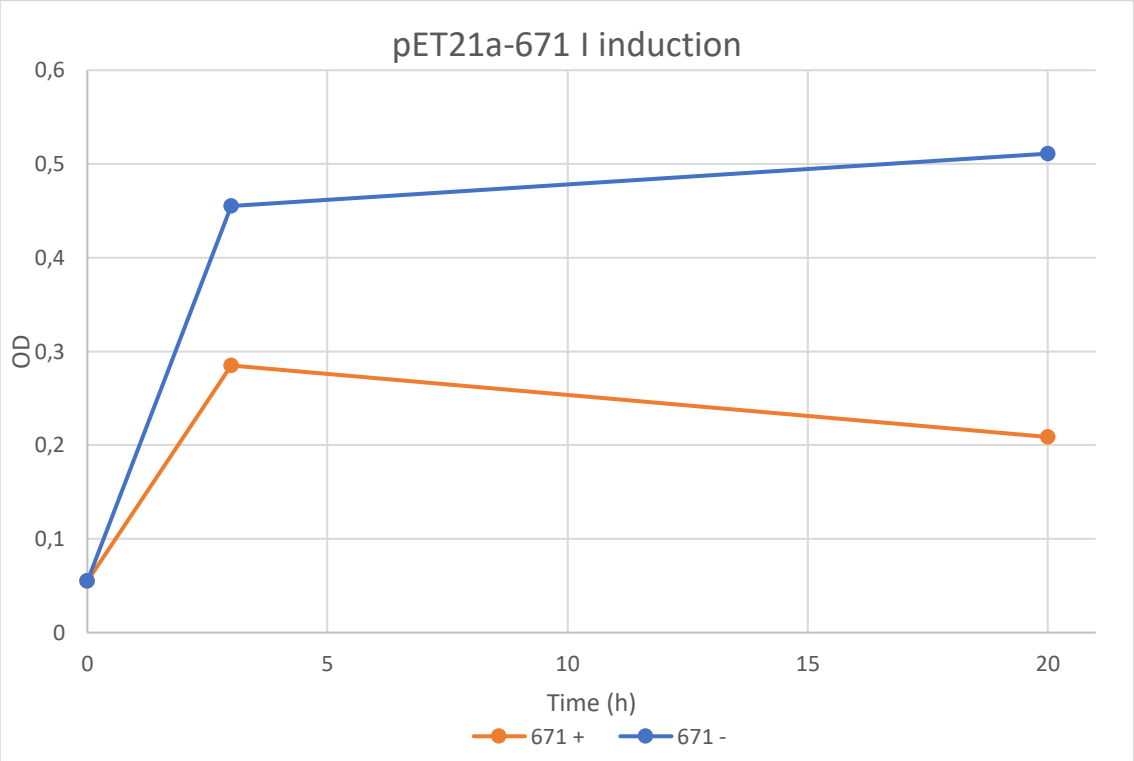

Figure 4.7: ***Optical density measurements along time of induction.*** 671+: culture induced with 1mM IPTG. 671-: culture not induced (control)

Retrieved samples were loaded into SDS/PAGE gel as is described in section 3.8. Samples were run at 80 mV for approximately 2 hours (figure 4.8). Expected protein is of 26,6 kDa, according to SeqBuilder software from Lasergene's DNAStar.

As it is depicted in figure 4.8, it is possible to discern an accentuated band in the induced samples of pET21a-671 I. Considering the weight of said band, which stands above the 25 kDa ladder mark, it can be correlated to the successful expression of protein 671.

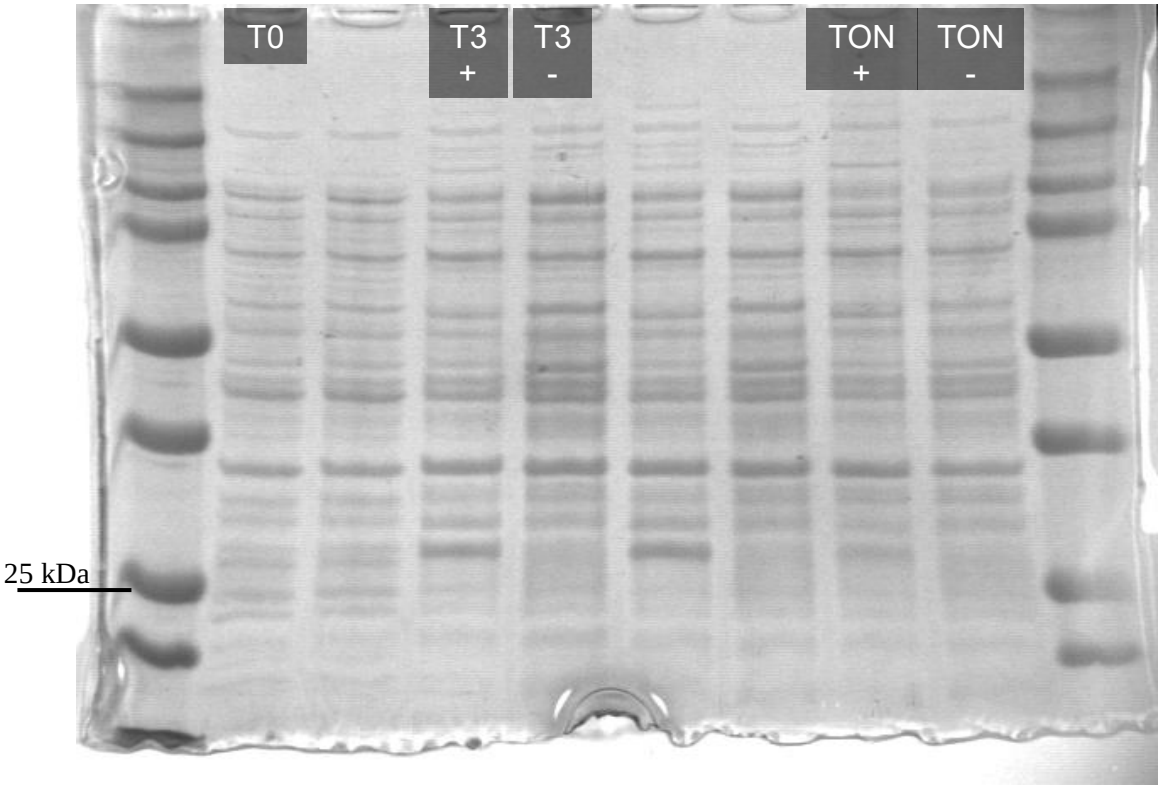

*Figure 4.8:**SDS/PAGE gel of pET21a-671 I protein induction test.*** T0: time 0 of induction. T3+: time 3-hour induced. T3-: time 3-hour non-induced. TON+: time overnight induced. TON-: time overnight non-induced.

4.4 Sequencing of pET21a-671 I

Sanger sequencing of plasmid pET21a-671 I was also performed in order to verify coherence with the *in-silico* sequence (section 3.9).

*Figure 4.9:**Sequencing of pET21a-671 I.*** A- Segment of sequencing traces assembled by SeqMan software from Lasergene's DNAStar and provided by STABvida laboratories. B: Segment of *in-silico* sequence depicting the deleted nucleotides.

As it is demonstrated in figure 4.9, sanger sequencing exposed an interruption of approximately 40bp in plasmid pET21a-671 I. This interruption implied the deletion of the conserved RBS of pET21a as well as the start codon, which could potentially hinder the expression of the protein.

The cause of this deletion was due to the digestion of the cloning vector with the XbaI enzyme instead of the NheI enzyme (figure 4.9 B). Digestion with enzyme NheI and EagI would not interfere with the RBS and start codon.

4.5 Construction of pET21a-671 II

Similarly to the procedure in constructing plasmid pET21a-671 I, the new plasmid was constructed by digesting with restriction enzyme NheI and EagI (as it was described in section 3.4) and ligated to the digested insert 671. Ligation of the XbaI /EagI digested 671 fragment with NheI/EagI digested pET21a is only possible because XbaI and NheI possess compaible cohesive ends. As it was shown in figure 4.2, NheI enzyme has a single restriction site that will linearize digested plasmid.

A PCR was performed to verify pET21a-671 II ligation, demonstrated in figure 4.10. As it was intended, samples of ligated pET21a-671 II (lanes 1, 2 and 3) display amplification of a DNA fragment of approximately 850bp which corresponds to the inserted gene 671. Lane 4 displays a band of roughly 300bp, which coincides with the expected amplification of auto-ligated pET21a. The existence of amplification in digested pET21a implies that digestion with either NheI or EagI was incomplete.

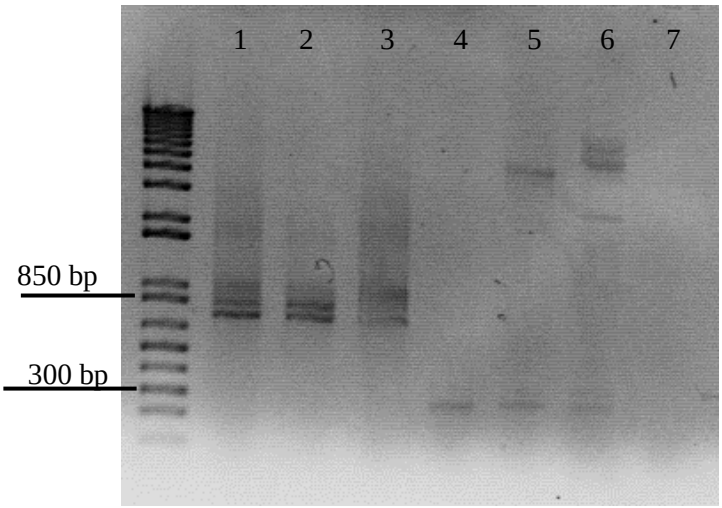

Figure 4.10: ***Cloning PCR of pET21a-671 II plasmid.*** Expected size of amplified fragment is approximately 850 bp, which verifies a proper insertion of gene 671. 1- PCR product of pET21a-671 II 10µL. 2- PCR product of pET21a-671 II 7µL. 3- PCR product of pET21a-671 II 5µL. 4- PCR product of auto-ligated pET21a. 5- PCR product of pET21a digested with NheI and EagI. 6- PCR product of pET21a. 7- Control.

4.6 Positive colonies of pET21a-671 II.

The confirmed ligated pET21a-671 II was transformed into *E.coli* strain Dh5α according to the protocol described in section 3.5. The auto-ligated pET21a was also transformed. Several

of the resulting colonies were stricken into fresh plates and screened via Colony Screening PCR (section 3.6)

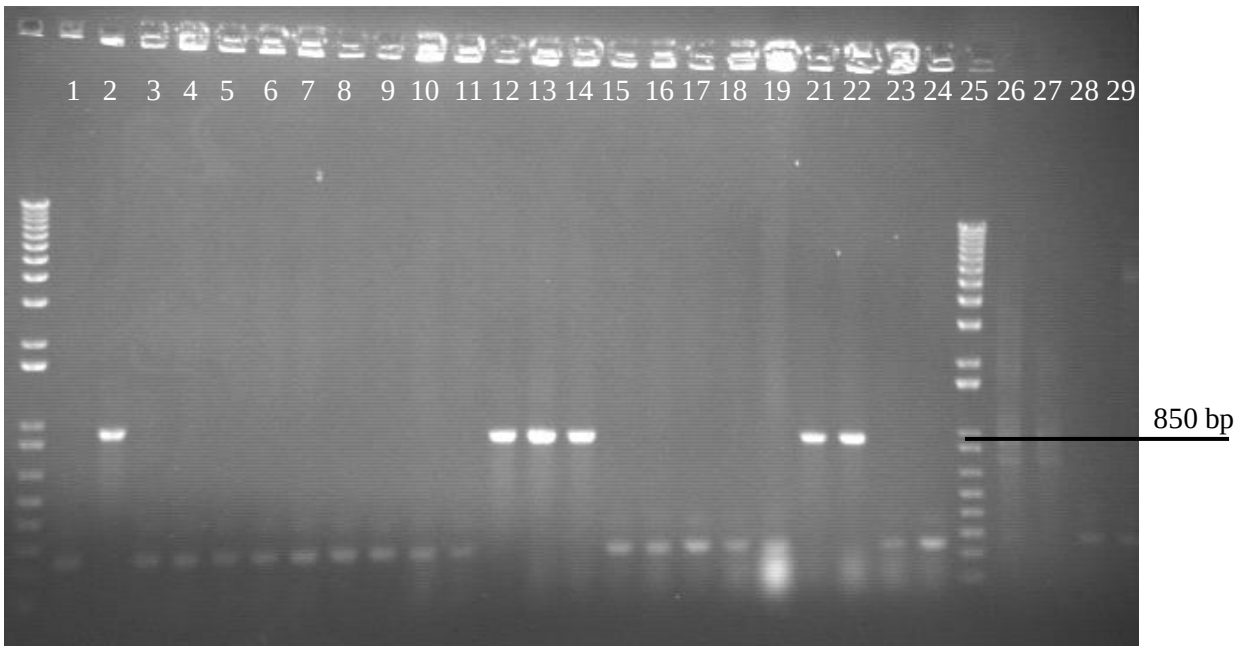

Figure 4.11: ***Colony Screening PCR of pET21a-671 II.*** 1 to 23- Amplification from transformed Dh5α with pET21a-671 II. 24- Amplification from transformed Dh5α with auto-ligated pET21a. 25- DNA Ladder. 26- Amplification from pET21a-671 II 10μL (positive control). 27- Amplification from pET21a-671 II 7μL (positive control) 28- Amplification from auto-ligated pET21a (negative control). 29- Amplification from pET21a.

PCR conditions and Master Mix constitution were as it is described in section 3.6. From the selected colonies transformed with pET21a-671 II screened via PCR, six displayed amplification of 800bp DNA fragment respective to gene 671 (figure 4.11). It was selected four of the positive colonies to proceed and performed a Digestive Profile with NaeI enzyme on two samples, positives A and B. Plasmid pET21a was also digested as negative control.

As it was demonstrated in figure 4.5, NaeI enzyme possesses three restriction sites digesting plasmid pET21a-671 II in three DNA fragments (table 4.2)

Table 4.2: ***Size comparison (in bp) of DNA fragments produced by NaeI digestion.***

pET21a	**pET21a-671 II**
3336	3336
1588	1588
447	***1197***

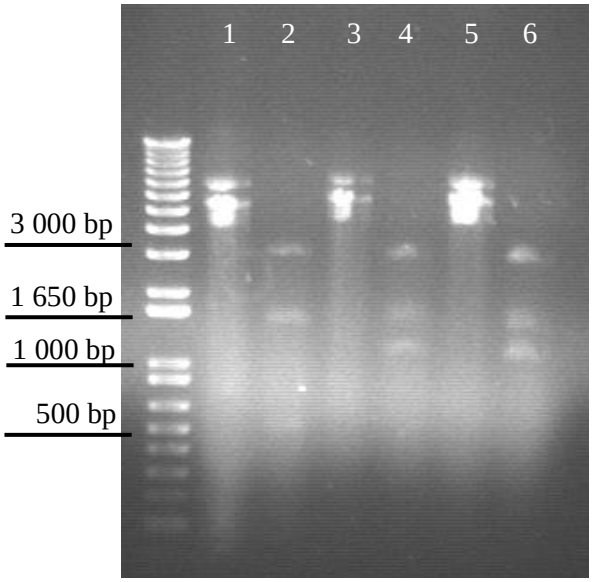

Figure 4.12 ***Digestive profile of pET21a-671 II by NaeI..*** 1- pET21a non-digested. 2- Digested pET21a. 3- pET21a-671 II A non-digested. 4- Digested pET21a-671 II A. 5- pET21a-671 II B non-digested. 6- Digested pET21a-671 II B.

The agarose gel in figure 4.12 depicts the digestion of the three plasmids by NaeI. As it was expected, all digested plasmids display two bands of equal size: a first with 3000bp followed by the second close to the 1650bp ladder band.

The last band of sample pET21a is barely visible in the agarose gel but is clearly below the 1000bp ladder mark. The positive samples pET21a-671 II A and B, however, exhibit the third band above the 1000bp which confirms the insertion of gene 671.

4.7 Induction test of pET21a-671 II A and B

Both positive plasmids pET21a-671 II were transformed into *E.coli* strain BL21(DE3) as it was described in section 3.5. One colony of each transformation was selected and inoculated in LB medium supplemented with 100μg/ml Ampycilin, incubated overnight at 37ºC, 150rpm.

Protein induction test was performed according to section 3.7, with 1mM IPTG and temperature 37ºC. Aliquots were retrieved every 30 minutes up to hour 4 of induction and the last one at overnight induction. Optical density was also measured at the stated points.

Retrieved samples were loaded into SDS/PAGE gel as is described in section 3.8. Samples were run at 80 mV for approximately 2 hours (figure 4.13). Expected protein is of 26,6 kDa, according to SeqBuilder software from Lasergene's DNAStar.

As it is depicted in figure 4.13, it is possible to discern an accentuated band in the induced samples of pET21a-671 II A. Considering the weight of said band, which stands above the 25 kDa ladder mark, it can be correlated to the successful expression of protein 671.

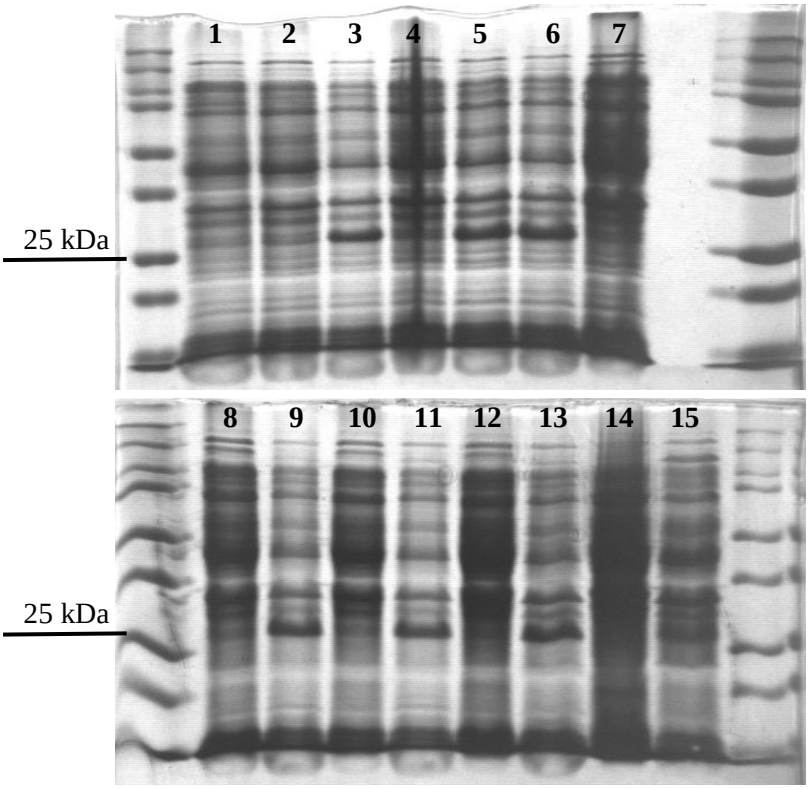

Figure 4.13: ***SDS/PAGE gel of pET21a-671 II A protein induction test.*** 1- Time 0:00. 2- Time 0:30 non-induced. 3- Time 0:30 induced. 4- Time 1:00 non-induced. 5- Time 1:00 induced. 6- Time 1:30 induced. 7- Time 1:30 non-induced. 8- Time 2:00 non-induced. 9- Time 2:00 induced. 10- Time 2:30 non-induced. 11- Time 2:30 induced. 12- Time 3:00 non-induced. 13- Time 3:00 induced. 14- Time ON non-induced. 15- Time ON induced.

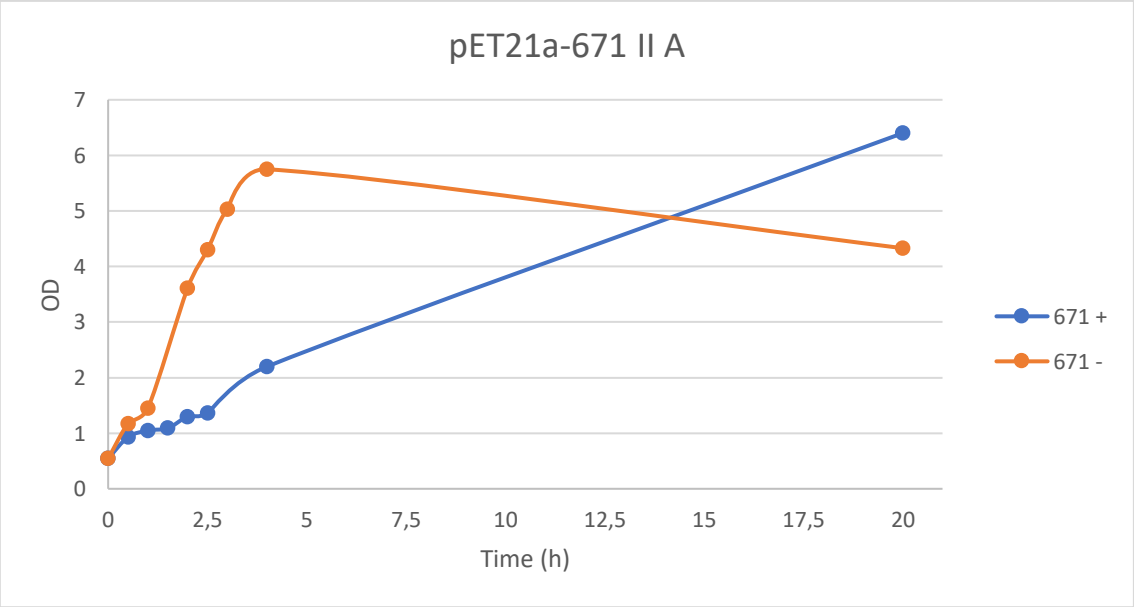

Figure 4.14 ***Optical density measurements along time of induction.*** 671+: culture induced with 1mM IPTG. 671-: culture not induced (control)

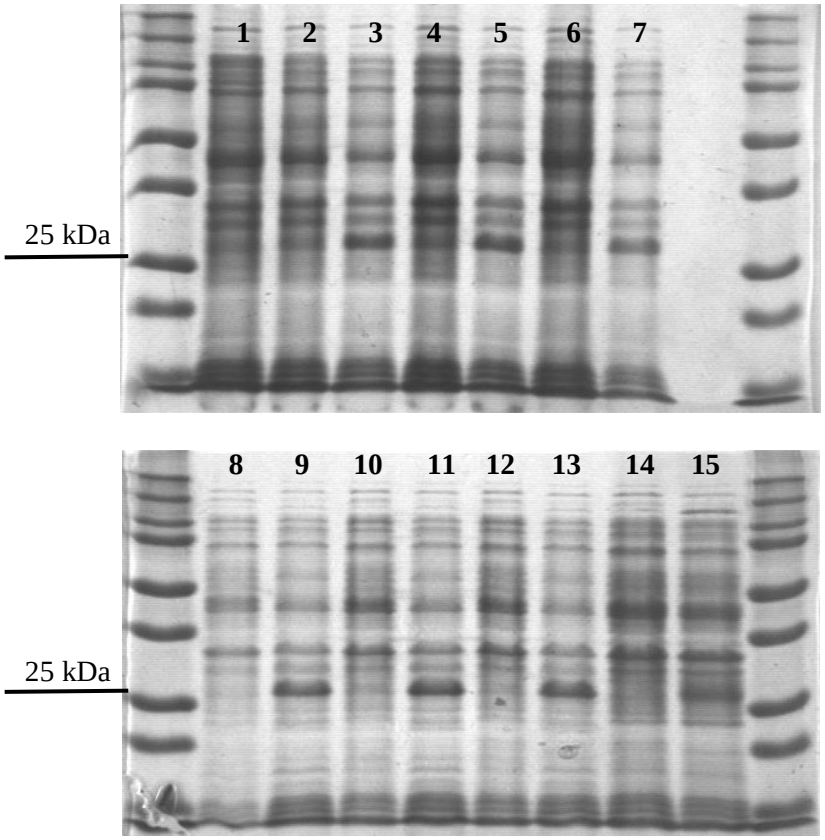

Figure 4.15: ***SDS/PAGE gel of pET21a-671 II B protein induction test.*** 1- T 0:00. 2- T 0:30 non-induced. 3- T 0:30 induced. 4- T 1:00 non-induced. 5- T 1:00 induced. 6- T 1:30 non-induced. 7- T 1:30 induced. 8- T 2:00 non-induced. 9- T 2:00 induced. 10- T 2:30 non-induced. 11- T 2:30 induced. 12- T 3:00 non-induced. 13- T 3:00 induced. 14- T ON non-induced. 15- T ON induced.

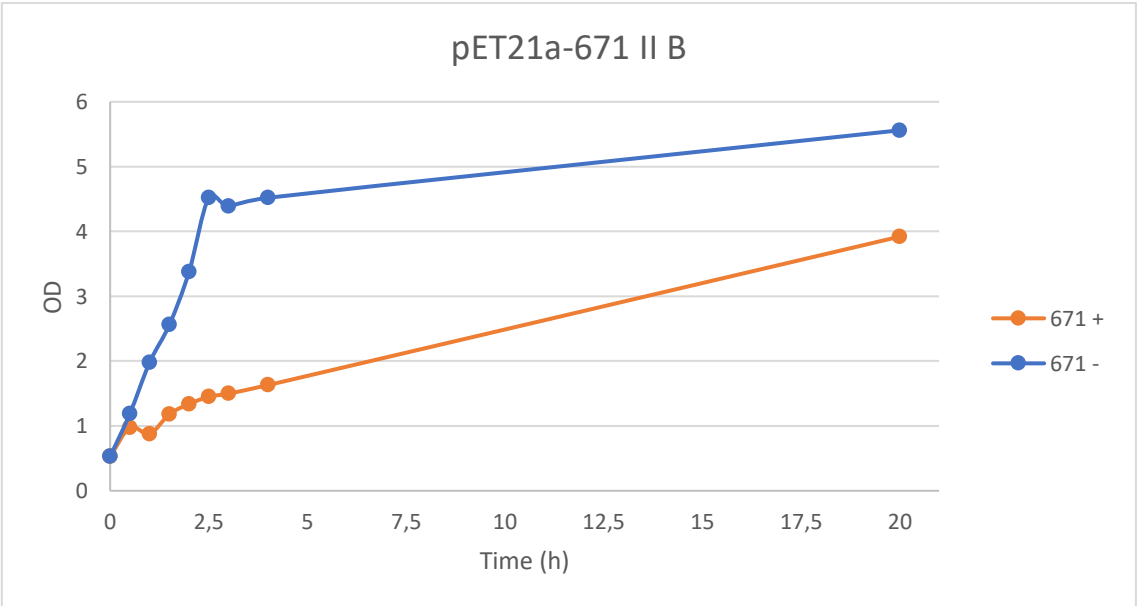

Figure 4.16: ***Optical density measurements along time of induction.*** 671+: culture induced with 1mM IPTG. 671-: culture not induced (control).

4.8 Sequencing of samples pET21a-671 II A and B

Sanger sequencing of plasmid pET21a-671 I was also performed in order to verify coherence with the *in-silico* sequence. Commercial primers *T7 promoter* and *T7 terminator* were used. Provided traces were assembled in Lasergene's DNAStar software, SeqMan.

Assembly of sample A with *in-silico* sequence showed a 100% match and 99% match with primers *T7 promoter* and *T7 terminator* respectively, and coherence was verified.

However, assembly of sample B with *in-silico* sequence displayed an interruption of 1 nucleotide in both provided traces, which implies a frame-shift in the plasmid sequence. This frame-shift results in the loss of the original stop codon and, consequentially an extension of approximately 30bp.

4.9 Additional inductions of pET21a-671 II A

After the first induction test validated plasmids pET21a-671 II A, pET21a-671 II B and pET21a-671 I it was performed a new assay in order to compare protein expression between the three constructs. Despite the variations revealed by sanger sequencing, all plasmids have successfully expressed protein in the initial experiments.

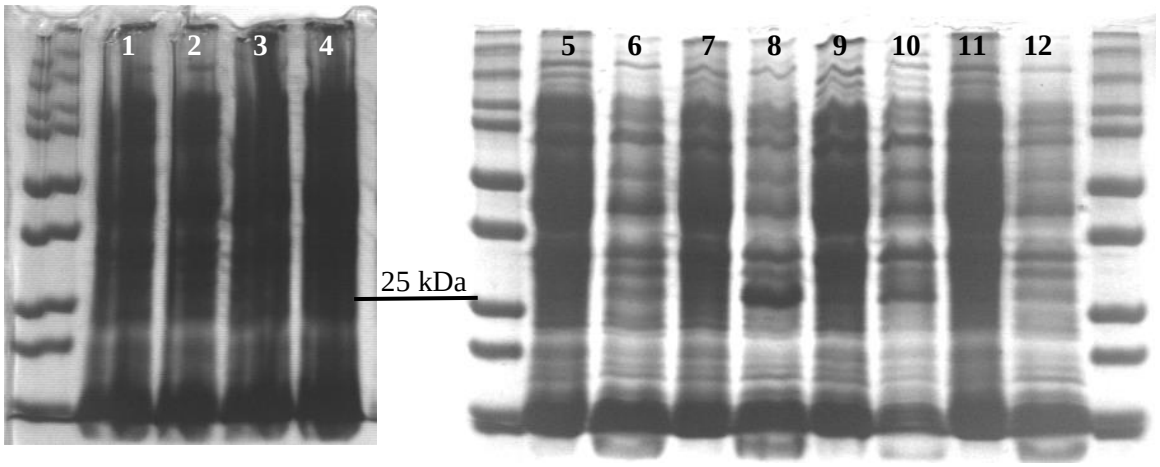

Figure 4.17: ***SDS/PAGE gel of protein 671 induction.*** 1- Time 0 pET21a-671 II A. 2- Time 0 pET21a-671 II B. 3- Time 0 pET21a-671 I. 4- Time 0 pET21a. 5- Time 3 hours pET21a-671 II A non-induced. 6- Time 3 hours pET21a-671 II A induced. 7- Time 3 hours pET21a-671 II B non-induced. 8- Time 3 hours pET21a-671 II B induced. 9- Time 3 hours pET21a-671 I non-induced. 10- Time 3 hours pET21a-671 I induced. 11- Time 3 hours pET21a non-induced. 12- Time 3 hours pET21a induced.

Transformation of BL21(DE3) and protein induction test were performed according to section 3.5 and 3.7, respectively. Aliquots were retrieved at time 0 of induction and after three hours (T3) of induction and loaded into SDS/PAGE gel prepared as is described in section 3.8.

Samples were run at 80 mV for approximately 2 hours (figure 4.17). Expected protein is of 26,6 kDa, according to SeqBuilder software from Lasergene's DNAStar.

As it is depicted in figure 4.17, induced samples of pET21a-671 II B and pET21a-671 I display an accentuated band superior to the 25 kDa ladder mark which can be correlated to protein 671. The band displayed in pET21a-671 I, however, is significantly less noticeable which can be explained by the loss of the RBS.

On contrary to previous results, sample pET21a-671 II A demonstrates no induction whatsoever.

Transformed colonies of BL21(DE3) were stricken and screened via Colony Screening PCR in order to verify successful transformation with pET21a-671 II A. Colony Screening PCR performed according to section 3.6

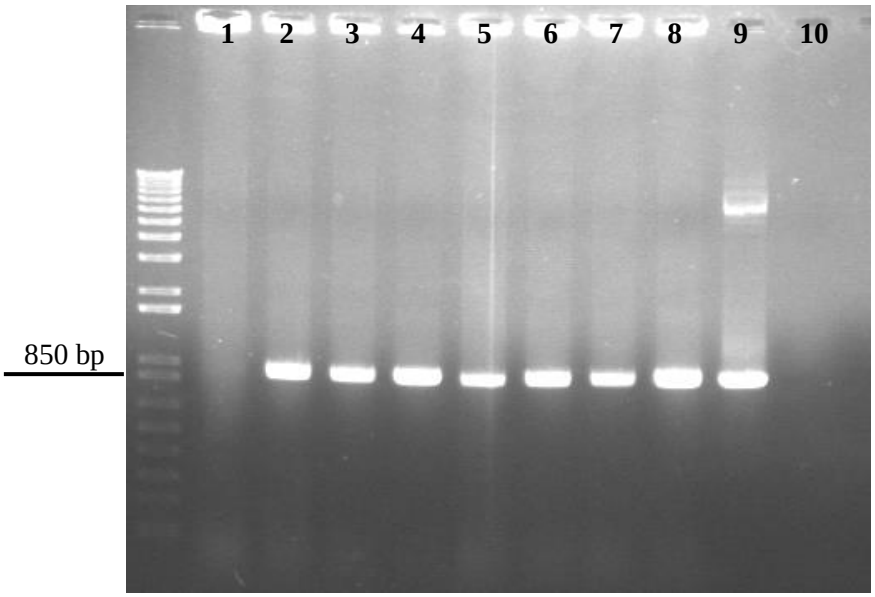

Figure 4.18: ***Colony Screening PCR of pET21a-671 II A.*** 1 to 8- Amplification from transformed BL21(DE3) with pET21a-671 II A. 9- Amplification from pET21a-671 II 10µL (positive control). 10- Control

As it is demonstrated in figure 4.18, all stricken BL21(DE3) colonies successfully amplified an 850bp DNA fragment that corresponds to gene 671 insert. Three colonies were selected and inoculated into fresh medium to perform an induction test.

Induction test for the three colonies followed conditions stated in section 3.6, with 1mM IPTG and temperature 37ºC. Aliquots retrieved at time 0 (T0) and at hour 3 (T3) of induction for all samples and loaded in SDS/PAGE gel (section 3.8, depicted in 4.19).

Similarly to the previous induction assay (figure 4.17), none of the three colonies selected of pET21a-671 II A successfully induced protein 671 (as is demonstrated in figure 4.19), despite PCR screening confirming a positive transformation.

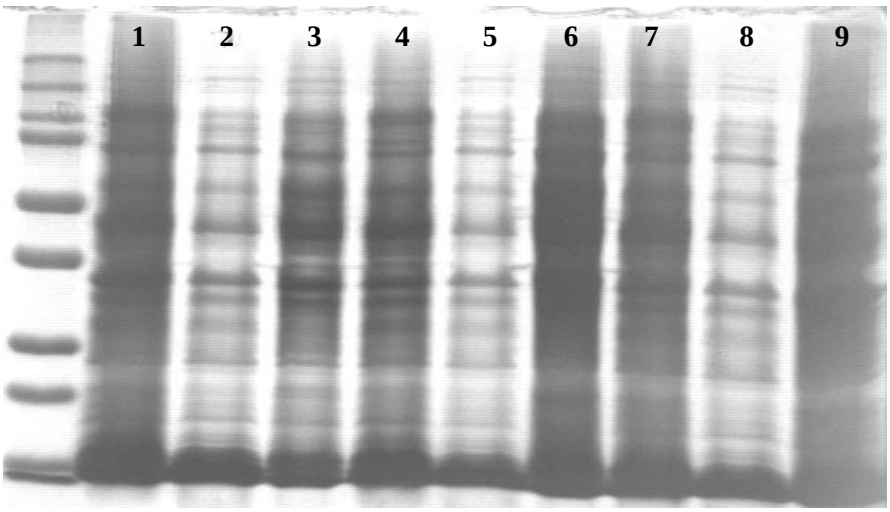

Figure 4.19: ***SDS/PAGE gel of pET21a-671 II A induction.*** 1- Time 0 hour Colony 1. 2- Time 3 hours induced Colony 1. 3- Time 3 hours non-induced Colony 1. 4- Time 0 hour Colony 2. 5- Time 3 hours induced Colony 2. 6- Time 3 hours non-induced Colony 2. 7- Time 0 hours Colony 3. 8- Time 3 hours induced Colony 3. 9- Time 3 hours non-induced Colony 3.

A series of factors could explain the unsuccessful induction of pET21a-671 II A. The concentration of IPTG added to the samples could be less than sufficient to induce expression of the protein. However, in all elaborated assays was used the same concentration of IPTG, in particular in the first assay (figure 4.13), where a successful expression of the protein was achieved in all induced samples. Similarly, toxic concentrations of IPTG could also explain the absence of expression, however, plasmids pET21a-671 II B and pET21a-671 I exhibited protein expression in all assays (figure 4.15 and 4.17). In all assays, the IPTG used was retrieved from the same stock and administered in equal concentrations to all cultures, which deems IPTG as the unlikely cause of this occurrence.

The use of abnormal BL21(DE3) competent cells could justify the inability to properly express the 671 protein. However, all culture samples of BL21(DE3) used in all induction assays were originally provenient from the same batch, prepared per point 3.1. Successful induction observed in samples pET21a-671 II B and pET21a-671 I (4.15 and 4.17) confirms proper activity of competent cells.

Another possible cause could be the contamination of the stock of pET21a-671 II A due to improper handling. All pET21a-671 II A transformations of BL21(DE3) were performed with extracted plasmid from the aforementioned stock. In figure 4.18 is displayed a Colony Screening of transformed pET21a-671 II A colonies with amplified 671 gene. Furthermore, re-sequence of

the multiple cloning site of the same plasmid confirmed no alterations occurred to the target sequence.

The occurrence of alterations on the remaining plasmid that could undermine the induction process is, however, plausible. To assess this hypothesis, it should be performed a PCR screening with different sets of primers that will amplify different areas of the plasmid.

4.10 Acquiring positives of pMAD del 671

Plasmid pMAD del 671 was constructed as it is described in sections 3.11 and 3.12. Transformation to *E.coli* strain DC10b was performed according to section 3.5 and selected colonies were screened via Colony Screening PCR with the primers pMAD1 and pMAD2. PCR conditions were as stated in section 3.6.

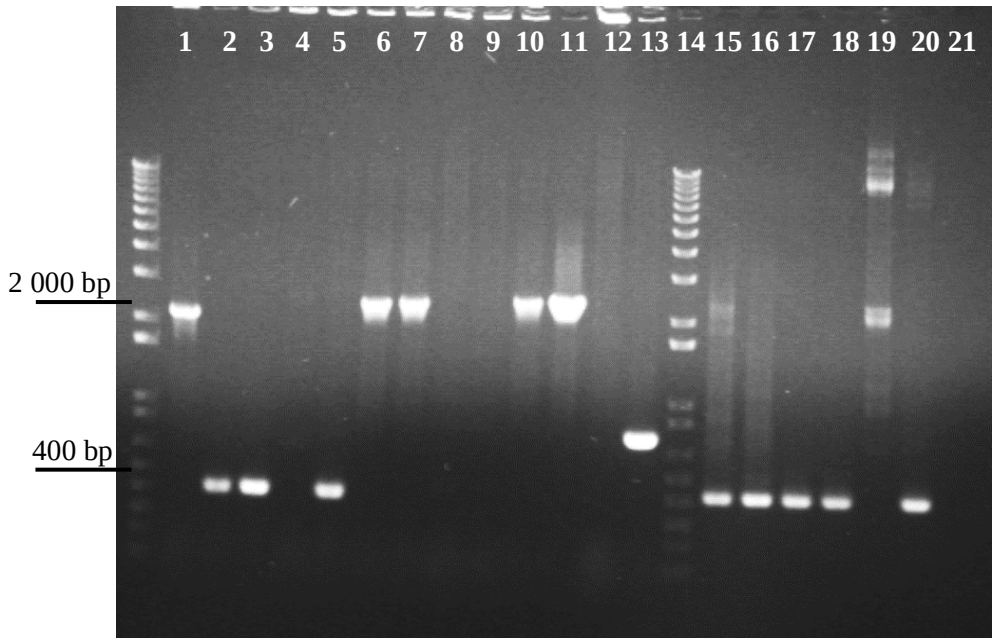

Figure 4.20: ***Colony Screening PCR of pMAD del 671.*** 1 to 13- Amplification from transformed DC10b with pMAD del 671. 14 DNA Ladder 15- Amplification from pMAD del 671 (positive control). 16- Amplification from auto-ligated pMAD. 17- Amplification from pMAD digested with BamHI. 18- Amplification from pMAD digested with EcoRI. 19- Amplification from pMAD del Sle1. 20- Amplification from pMAD. 21- Control.

A positive colony of pMAD del 671 will amplify a DNA fragment of 2000bp (which corresponds to the combined 1000bp Up region and 1000bp Down region). In figure 4.20, it is possible to discern five DC10b colonies that display a 2000bp band amplification similarly to the positive control pMAD del 671 (lane 1). Lanes 2, 3, 4 and 6 display a band of approximately 400bp which coincides to the amplification of the auto-ligated pMAD. The existence of

amplification product in digested pMAD plasmid implies that digestion with either BamHI or EcoRI was incomplete.

Colonies positive for pMAD del 671 were stricken into fresh plates and plasmid DNA was extracted using a Miniprep kit. It followed a digestion with restriction enzyme AvaI in order to assess and Digestive Profile. Digestion with AvaI enzyme will result in two DNA fragments with the following sizes.

Table 4.3: ***Size comparison (in bp) of DNA fragments produced by AvaI digestion***

pMAD	**pMAD del 671**
9202	9202
482	***2488***

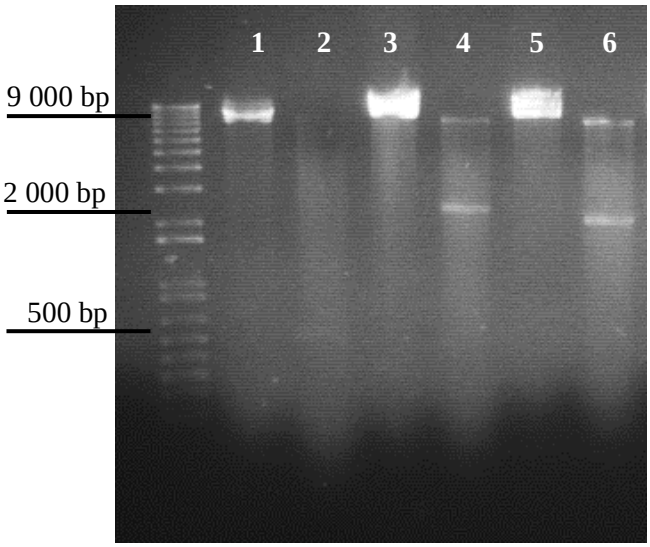

Figure 4.21: ***Digestive Profile of pMAD del 671 by AvaI.*** 1- pMAD non-digested. 2- Digested pMAD. 3- pMAD del 671 non-digested. 4- Digested pMAD del 671. 5- pMAD del 671 B non-digested. 6- Digested pMAD del 671 B.

Figure 4.21 depicts the digestion of three plasmids by enzyme AvaI. All plasmids display a band correspondent to the 9202bp DNA fragment. As expected, digested pMAD displays a second band of approximately 500bp albeit barely visible. The remaining digested samples of pMAD del 671 exhibit a second band above the 2000bp ladder size, which confirms the selected positives.

4.11 Sequencing of pMAD del 671

Sanger sequencing of plasmid pMAD del 671 was also performed in order to verify coherence with the *in-silico* sequence. Primers NCTC671UpFwEcoRI (P1) and NCTC671DwRevBamHI (P4) were used as forward and reverse primers, respectively. Provided traces were assembled in Lasergene's DNAStar software, SeqMan.

Assembly of pMAD del 671 traces with *in-silico* sequence showed 99% match with both primers and coherence was verified.

4.12 Integration of pMAD del 671 in NCTC 8325-4 genome

After verified and sequenced positives of pMAD del 671, it followed the electroporation of *S.aureus* strain RN4220, as demonstrated in section 3.13. Several colonies were selected for phage transduction (section 3.14), at the end of which was obtaining the positives NCTC 8325-4 + pMAD del 671.

It followed the gene Knock-out protocol as it is demonstrated in section 3.15. Verification of integrated pMAD del 671 was verified via PCR screening from the extracted genomic DNA (sections 3.17 and 3.16)

Samples were screened with two different sets of primers that will allow to determine in which region, Up or Down, integration occurred. In table 4.4 it is demonstrated the obtained amplification fragments per each pair of primers. For a given sample, the amplification of a fragment of 3000bp with the set P1-pMAD2 and the amplification of a fragment of 2000bp with the remaining set, will indicate the integration of plasmid pMAD del 671 occurred on the Down region.

Table 4.4: ***pMAD del 671 integration according to each set of primer.***

	P1 – pMAD2	**pMAD1 – P4**
Integration Down	3000bp	2000bp
Integration Up	2000bp	3000bp
No integration	2000bp	2000bp

In figure 4.22 is depicted the agarose gel of the integration PCR screening. Samples were loaded in gel following the sequence P1-pMAD2 sample / pMAD1-P4 sample, in order to facilitate comparison of the resulting amplifications.

Sample pair 1 displays two amplification bands of approximately 2000bp which is expected when no integration occurs. In sample pair 2 we see a band of approximately 3000bp amplified

by primer set pMAD1-P4 which corresponds to Up-region integration. Sample pair 3 shows a band of 3000bp, which would suggest integration, but also a second band of 2000bp. This result proposes the extracted DNA was retrieved from mixed colonies.

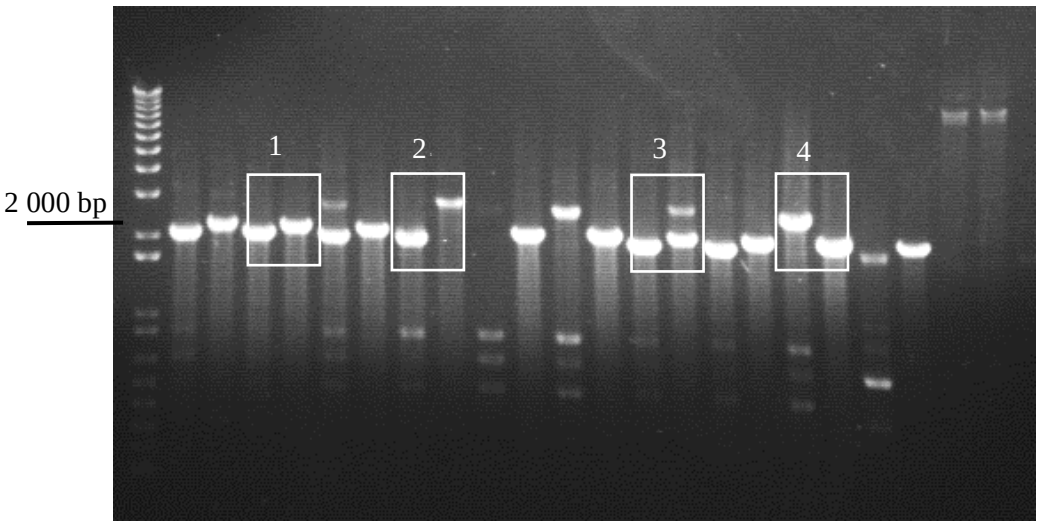

Figure 4.22: ***Integration Screening of pMAD del 671.*** 1- amplification pair negative integration. 2- amplification pair positive for Up-region integration. 3- amplification pair with mixed colonies. 4- amplification pair positive for Down-region integration.

Sample pair 4 displays a band of 3000bp amplified by primer set P1-pMAD2, which corresponds to Down-region integration.

Four colonies NCTC 8325-4 that displayed positive integration (one displayed integration by Up-region, and the remaining 3, Down-region integration) were selected and stricken to fresh plates.

4.13 Excision of pMAD del 671

After confirmation of positive integration, it followed the excision procedure. Similarly to the integration step, positive excised colonies were confirmed via PCR screening described in section 3.17.

Samples were screened with primers forward NCTC671UpFwEcoRI (P1) and reverse NCTC671DwRevBamHI (P4). These primers will amplify the DNA fragment that consists of 1000bp Upstream region and 1000bp Downstream region of gene 671.

In NCTC 8325-4 wildtype, these primers will also amplify gene 671, so the expected size of the amplified fragment will be of 2000bp from regions Up and Down plus gene 671, which amounts to approximately 3000bp fragment. In a successful excision of gene 671, the amplified product will consist of a DNA fragment of 2000bp.

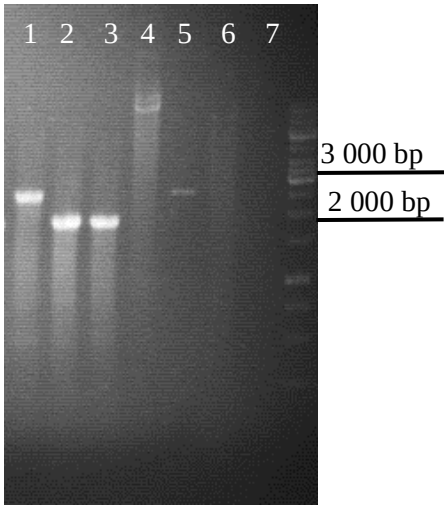

Figure 4.23: ***PCR screening of NCTC Δ671.*** 1- amplification from screened sample A. 2- amplification from screened sample B. 3- amplification from screened sample C. 4- amplification from pMAD del 671. 5- amplification from NCTC 8325-4 wildtype. 6- amplification from pMAD. 7- control.

In figure 4.23 is demonstrated the agarose gel of the PCR screening of samples A, B and C. Sample A displays a band of approximately 3000bp which corresponds to the amplified fragment Up-671-Down and is, therefore, a negative excised sample.

Samples B and C display a band of 2000bp which is the expected size amplified from a positive sample, which consists of fragments Up-Down.

In total, ten confirmed NCTC 8325-4 Δ671 mutants were acquired.

5 Conclusion

Both plasmids of pET21a-671 and pMAD del 671 were successfully constructed. PCR screening with the primers *T7 promoter* and *T7 terminator* confirmed positive insertion of gene 671 in the MCS of vector pET21a. Similarly, screening with the primers pMAD1 and pMAD2 confirmed proper insertion of fragment Up-Down in the vector pMAD.

The first vector for protein expression was pET21a-671 I, acquired by plasmid digestion with enzymes XbaI and EagI. Sequencing, however, revealed an interruption of approximately 40bp when assembled with the *in-silico* sequence. The cause disruption, that resulted in the loss of the RBS and the start codon in the plasmid, was determined to be the erroneous digestion with enzyme XbaI, in place of NheI. Despite the loss of the RBS and start codon, this plasmid managed a successful protein expression.

The second vector, pET21a-671 II, was acquired by plasmid digestion with the proper digestion with enzymes NheI and EagI. Of the obtained positives, two were selected and tested for protein induction, pET21a-671 II A and pET21a-671 II B. Sequencing revealed the loss of ORF in plasmid B that would result in the expression of a longer protein. No mismatched were found in plasmid A. Both plasmids were able to successfully express protein in the first induction assay, however, repetitions of the assay with plasmid A were unsuccessful. Due to these inconsistencies in the expression of plasmid pET21a-671 II A, purification of the protein 671 was not accomplished. The reasons behind the loss of expression ability of plasmid are yet to fully assessed.

Future work will consist in the purification of protein 671 from a third pET21a-671 II positive, plasmid C, followed by the assessment of the protein enzymatic profile by zymography.

Mutants NCTC 8325-4 Δ671 were obtained and confirmed via PCR screening. It follows the characterization of the mutant by assessing cell growth rate comparatively to wildtype, and cell morphology.

6 References

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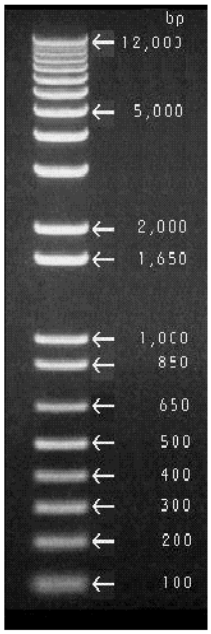
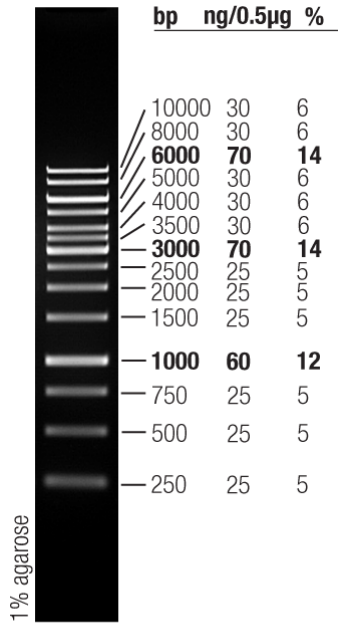
Anexes

A. 1 Kb Plus DNA Ladder (Thermo Scientific)



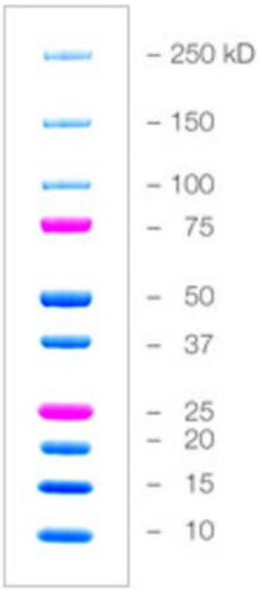
1 Kb PLUS DNA LADDER
0.7 µg/lane
0.9% agarose gel

B. GeneRuler 1kb DNA Ladder (Thermo Scientific, catalog number: SM0312)



0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 45 min

C. Precision Plus Protein Dual Color Standarts (PPPS) (Bio-Rad Laboratories, catalog number: 161037)



D. Miniprep Kit: Wizard Plus SV Minipreps DNA Purification Systems (Promega Corporation, catalog number: A1465)

Centrifugation Protocol

Production of Cleared Lysate

1. Pellet 1–10ml of overnight culture for 5 minutes.

2. Thoroughly resuspend pellet with 250µl of Cell Resuspension Solution.

3. Add 250µl of Cell Lysis Solution to each sample; invert 4 times to mix.

4. Add 10µl of Alkaline Protease Solution; invert 4 times to mix. Incubate 5 minutes at room temperature.

5. Add 350µl of Neutralization Solution; invert 4 times to mix.

6. Centrifuge at top speed for 10 minutes at room temperature.

Binding of Plasmid DNA

7. Insert Spin Column into Collection Tube.

8. Decant cleared lysate into Spin Column.

9. Centrifuge at top speed for 1 minute at room temperature. Discard flowthrough, and reinsert Column into Collection Tube.

Washing

10. Add 750µl of Wash Solution (ethanol added). Centrifuge at top speed for 1 minute. Discard flow through and reinsert column into Collection Tube.

11. Repeat Step 10 with 250µl of Wash Solution.

12. Centrifuge at top speed for 2 minutes at room temperature.

Elution

13. Transfer Spin Column to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column. If the Spin Column has Column Wash Solution associated with it, centrifuge again for 1 minute at top speed, then transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube.

14. Add 100µl of Nuclease-Free Water to the Spin Column. Centrifuge at top speed for 1 minute at room temperature.

15. Discard column, and store DNA at –20°C or below

E. Clean Up Kit: Wizard SV Gel and PCR Clean-Up System (Promega Corporation, catalog number: A9285)

<u>Gel Slice and PCR Product Preparation</u>

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. ***Processing PCR Amplifications***

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA
1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing
4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution
7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.